IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
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Peter Gluckman *et al.*	)	Group Art Unit: 1654
	)	
Application No.: 10/606,745	)	Examiner: Jeffrey E. RUSSEL
	)	
Filed: June 27, 2003	)	Confirmation No.: 5345
	)	
For: IGF-1 TO IMPROVE NEURAL OUTCOME (As Amended)	)	

DECLARATION OF AZAD BONNI, M.D., Ph.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Azad Bonni, M.D., Ph.D., do hereby state:

1. I am currently an Associate Professor of Pathology at the Harvard Medical School. Prior to taking this post in 1999, I obtained my Medical Degree in 1986 from Queen's University in Kingston Ontario, Canada; and my Ph.D. in the subject of Neuroscience in 1996 from Harvard University.

2. I also trained in neurology from 1987 to 1990 at McGill University and became a fellow of the Royal College of Physicians and Surgeons of Canada in 1991 (FRCP (neurology)).

3. At Harvard Medical School, I am the Principal Investigator of a laboratory, "the Bonni Laboratory," where our research is focused on elucidating the mechanisms regulating neuronal morphogenesis and connectivity in the brain. Our research also investigates how abnormalities in the development of brain signaling pathways contribute to neurological disorders. Our

research employs a combination of molecular and cell biological, biochemical, and genetic approaches and uses neurons from rat and mouse brains in culture and *in vivo*.

4. I am the author of several peer-reviewed publications in the area of molecular and developmental neurobiology.

5. I have been asked to review U.S. Patent No. 5,714,460 ("the '460 patent"). I have been informed that the '460 patent has been submitted as a reissue application, U.S. Patent Application Serial No. 10/606,745 ("the '745 application"). I have been informed that the text of the '460 patent, other than the claims, is virtually the same as the text of the '745 application. I have reviewed pending Claims 16-26, 28-38, 64, and 65 as well as Applicants' proposed amended Claims 16, 28, and 64-77 in the '745 application.

6. I have been informed that the Examiner of the '745 application considers Claims 16-25 and 28-37 unclear due to the phrase "wherein said CNS injury predominantly affects glia." I have been informed that Applicants' proposed amended Claims 16, 28, and 66-77 in the '745 application also contain the phrase "wherein said CNS injury predominantly affects glia."

7. In my opinion, the phrase "wherein said CNS injury predominantly affects glia" would be readily understood by one of ordinary skill in the art.

8. I consider a person of ordinary skill in the art to be a person with an M.D. and/or Ph.D. in a biological science, and at least two years of experience in a laboratory working with cells of the Central Nervous System (CNS).

9. The CNS includes the brain and spinal cord and their respective components. The CNS is characterized by containing neurons and glia. Neurons are the cells responsible for information signaling, and can be further subdivided according to several characteristics including the type of neurotransmitter they produce. For instance, there are cholinergic neurons

that signal using the neurotransmitter acetylcholine and dopaminergic neurons that signal using the neurotransmitter dopamine.

10. Glia are non-neuronal cells, found in far greater numbers than neurons in the human CNS. Glia provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the central nervous system. Glia also form and maintain the blood-brain barrier and regulate the activity of neurons. Glia also play a key role in brain development.

11. In my opinion, a CNS injury that predominantly affects glia is a CNS injury that results in abnormalities mainly to the resident glial cells (which include astrocytes, oligodendrocytes, and ependymal cells) rather than to neurons.

12. There are known pathogenic CNS insults that predominantly affect glia. For example, demyelinating diseases (*see, e.g.*, Claim 9 of the '460 patent) lead to abnormalities predominantly in oligodendrocytes, which are the cells that form the myelin sheath around neurons. Thus, a demyelinating disease is an example of a CNS injury that predominantly affects glia. A common example of a demyelinating disease or disorder is Multiple Sclerosis ("MS"). *See Column 1, Lines 44-46 of the '460 patent.*

13. Other examples of CNS insults that can predominantly affect glia include periventricular leucomalacia, carbon monoxide inhalation, ammonia intoxication, and gaseous intoxication (as disclosed in the '460 patent at Column 1, Lines 24-28 and Lines 47-56). Attached hereto are excerpts from the TEXTBOOK OF NEUROPATHOLOGY (Richard L. Davis and David M. Robertson, Editors, 2nd Edition 1991) and from GREENFIELD'S NEUROPATHOLOGY (J. Hume Adams and Leo W. Duchen, Editors, 5th Edition 1992). Also attached hereto is J. Antel's "*Oligodendrocyte/myelin injury and repair as a function of the central nervous system environment,*" 108 CLINICAL NEUROLOGY AND NEUROSURGERY 245-249 (2006); R. Folkerth's

"*Periventricular LeukomalaciaL Overview and Recent Findings,*" 9 PEDIATRIC AND DEVELOPMENTAL PATHOLOGY 3-13 (2006); and R. B. Parkinson *et al.*'s "*White matter hyperintensities and neuropsychological outcome following carbon monoxide poisoning,*" 58 NEUROLOGY 1525-1532 (May 2002). The foregoing publications verify that these CNS insults can predominantly affect glia.

14. The text of the '460 patent clearly refers to treatment of CNS injury "wherein said CNS injury predominantly affects glia." To begin, the '460 patent explains in its Abstract that the invention pertains to "**[a] method of treating injuries to or diseases of the central nervous system that predominantly effects glia** and/or non-cholinergic neuronal cells characterized in that it comprises the step of increasing the active concentration(s) of insulin-like growth factor 1 and/or analogues thereof in the central nervous system of the patient." (emphasis added). The "Field of the Invention" at Column 1, Lines 10-19 of the '460 patent further states that "**[t]his invention relates to methods and therapeutic compositions for the treatment or prevention of central nervous system (CNS) damage** and relates particularly although not necessarily to a method of increasing the concentration of insulin-like growth factor 1 (IGF-1) in the central nervous system of the patient to treat an injury or disease **that primarily causes damage to glia** and/or other non-cholinergic cells of the CNS." (emphasis added).

15. In some passages in the '460 patent, by referring to "glia and/or other non-cholinergic cells," glia are effectively characterized as non-cholinergic cells in the CNS. This is true, because they are not neurons which use the neurotransmitter acetylcholine – indeed, they are not neurons at all. However in my opinion, by stating that certain CNS injuries or diseases primarily cause damage to "glia **and/or** other non-cholinergic cells," a person of ordinary skill in the art would have reviewed the list of disclosed injuries and diseases for those which can cause damage

19. I declare under penalty of perjury that the foregoing is true and correct. The foregoing statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful, false statement may jeopardize the validity of the application and any patent issued or reissued thereon.

Azad Bonni, M.D., Ph.D.

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CHAPTER 10

Exogenous Toxic-Metabolic Diseases Including Vitamin Deficiency

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INTOXICATIONS

Introduction

People are exposed daily to an ever-expanding array of toxic compounds. Many of these affect the nervous system either selectively or in association with involvement of other organ systems. Appreciation of the importance of neurotoxicity is reflected by the appearance in recent years of journals and monographs devoted exclusively to this aspect of toxicology. In this chapter, discussion is limited to selected exogenous toxins that are relatively common and/or known to produce morphologically demonstrable central nervous system lesions.

Hypoxia and Toxic Gases

Classification

Several intoxication states share, in common, deficient delivery and/or utilization of oxygen and substrate. The hypoxic effects of such intoxications are particularly apparent in the heart and brain. The latter normally receives about 15% of the cardiac output, consumes about 20% of the blood oxygen, and metabolizes about 10–20% of the blood glucose. The oxygen and substrate deficiencies have been variously classified. The following is a simplified but useful version:

1. Anoxic or hypoxic hypoxia results from the absence or insufficiency of inhaled oxygen. This may be due to impaired ventilatory activity or insufficient oxygen in inhaled gases, e.g., anesthetic accidents. It may also result from pulmonary disorders that prevent absorption of oxygen, e.g., pulmonary edema.
2. Anemic hypoxia results from a decrease in oxygen transport that is due to either anemia or a reduced capacity of the hemoglobin to transport oxygen, e.g., carbon monoxide poisoning.
3. Stagnant hypoxia results from reduction or cessation of blood flow that is due to reduced cardiac output or impaired local perfusion. The cerebral lesions seen with stagnant hypoxia actually reflect the combined effects of inadequate oxygen supply, inadequate glucose supply, and the accumulation of catabolites such as lactic acid.
4. Histotoxic hypoxia results from cellular intoxications that render the cells incapable of utilizing oxygen and substrate, e.g., cyanide intoxication which poisons the respiratory enzymes.

5. Oxyachrestic hypoxia results from hypoglycemia in which oxygen is not utilized because of the substrate deficiency.

Carbon Monoxide

Carbon monoxide is a colorless, odorless gas that is produced by the incomplete combustion of various fuels. The toxic effects result predominantly from impaired transport of oxygen. Hemoglobin combines reversibly with carbon monoxide but has about a 250-fold greater affinity for carbon monoxide than for oxygen. In addition, the carbon monoxide causes the oxygen that is bound to hemoglobin to be released less easily to other tissues. Therefore, a given degree of carboxyhemoglobinemia produces more severe tissue hypoxia than does a comparable degree of anemia. Still further toxic effects may result from the interaction of carbon monoxide with other hemoproteins, such as myoglobin, cytochrome oxidase, cytochrome P-450, catalases, and peroxidases. The magnitude of the toxicity from the combination with these other hemoproteins remains unknown.

The clinical manifestations of acute carbon monoxide intoxication can be correlated with the concentration of carboxyhemoglobin (percent saturation) in the blood. Thus they are influenced by both the duration of exposure and the concentration of carbon monoxide in the environment. Symptoms are referable to the tissues with the greatest oxygen demands, i.e., brain and heart. In general, a previously healthy individual will experience severe headache and dizziness at 20–30% saturation; impaired vision, hearing, and mental function at 40–50% saturation; coma and convulsions at 50–60% saturation; and cardiorespiratory failure and death at saturation over 70%. The toxicity may be markedly potentiated by preexisting cardiovascular disease. Many of the immediate acute deaths are the result of myocardial dysfunction.

Although carboxyhemoglobin has a distinctive bright red color, acutely intoxicated patients may appear flushed, pallid, or even cyanotic while still alive. The classical "cherry-red" color of skin, blood, and viscera is observed more often as a postmortem phenomenon. The skin may show extensive blister or bullae formation. Petechial hemorrhages, retinal hemorrhages, and pulmonary edema have also been observed. Brains from individuals dying within a few hours of the carbon monoxide intoxication generally show congestion and edema, an abnormal color, and rare petechial hemorrhages (Fig. 10.1). The cherry-red color of the fresh brain may become less apparent with prolonged formalin fixation. Brains from individuals dying 1–7 days after intoxication commonly show more extensive petechial hemorrhages. Pallidal necrosis is classically associated with delayed death from carbon monoxide intoxication. Occasionally, these lesions have been demonstrated antemortem by computerized tomography (91). Grossly discernible foci of pallidal necrosis are seen most often in individuals who survive for 6 or more days after the intoxication (53). Microscopic foci of necrosis and/or petechial hemorrhages develop sooner. Lapresle and Fardeau (53) observed pallidal necrosis in 16 of 22 patients who survived 1–139 days after intoxication. Rarely, the lesion is unilateral; more often, the lesions are merely asymmetrical, and bilateral lesions can be demonstrated in multiple planes of section. The necrosis most often involves the inner segment of the pallidum but may extend laterally into the outer segment or dorsally into the internal capsule (Fig. 10.2). It must be emphasized that the pallidal necrosis, while characteristic of delayed death from carbon monoxide, is not unique to this condition and has been seen in a wide variety of intoxications and anoxic states. The pathogenesis of the pallidal necrosis has been the subject of much debate over the years. Many authors regard pallidal necrosis as a manifestation of impaired circulation through the pallidal branches of the anterior choroidal arteries. Hemorrhagic and necrotic lesions of the cerebral cortex and Ammon's horn are also commonly encountered. Lapresle and Fardeau (53) found foci of cortical necrosis in

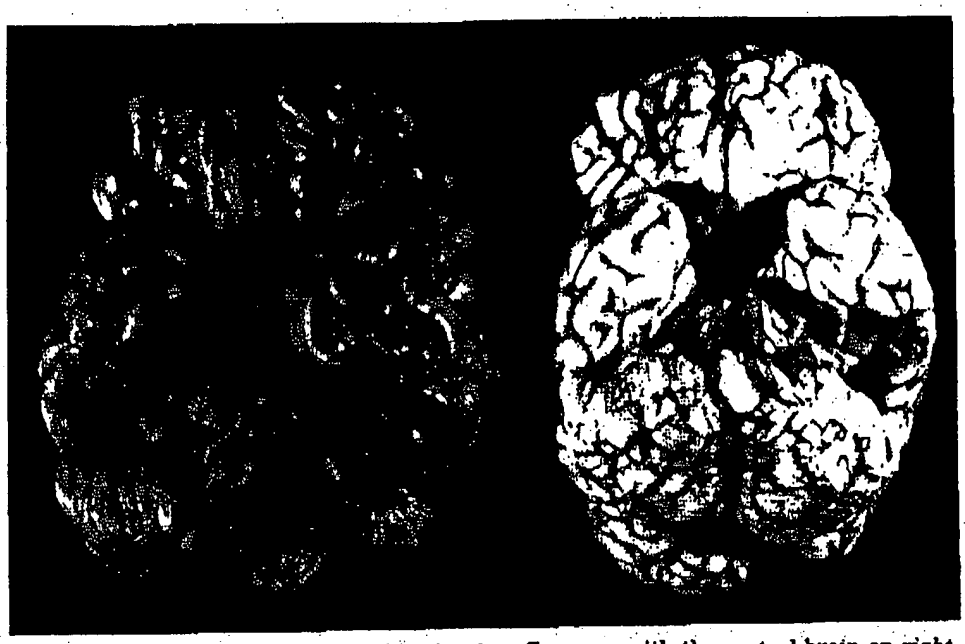

Figure 10.1. Acute carbon monoxide poisoning. Compare with the control brain on *right*.

Figure 10.2. Delayed death from carbon monoxide poisoning. Note the bilateral pallidal necrosis.

12 of their 22 cases and varying degrees of hippocampal injury in 10 of 20 cases. The cerebellum may show loss of Purkinje cells and loss of cells from the internal granular cell layer.

Lesions of the white matter are encountered equally as often and in association with gray matter lesions in individuals with delayed death from carbon monoxide intoxication. Four categories of white matter lesions have been delineated; however, there is much overlap among these groups (53). The first category consists of multiple small necrotic foci in the centrum semiovale (Fig. 10.3) and interhemispheric commissures. These small necrotic lesions are found predominantly in the anterior deep central white matter and anterior portion of the corpus callosum. The necrotic foci are centered about small blood vessels that contain swollen endothelial cells with vesicular nuclei. Some of the endothelial cells may even contain mitotic figures. The second category of lesions consists of extensive, confluent areas of necrosis. These necrotic zones may extend from the frontal to the occipital poles within the deep periventricular white matter and throughout the corpus callosum. The lesions are sharply demarcated and tend to spare the arcuate fibers. Histologically, the lesions show extensive axonal destruction and contain numerous lipid-laden macrophages. The third category of lesions consists of demyelination with relative preservation of axons in the deep periventricular white matter. The lesions may be small and discrete, extensive, or even confluent. Arcuate fibers tend to be spared. This third category is the type that is seen most often in patients with delayed deterioration or the so-called biphasic myelinopathy of Grinker. The fourth category consists of very small necrotic foci limited to the hemispheric white matter. The last category seems merely to be a restricted earlier form of the first category. As with the gray matter lesions, identical white matter lesions can be seen in other anoxic or hypoxic states (37). However, in the case of carbon monoxide poisoning, direct toxic effects on the white matter may be contributory to the development of the lesions.

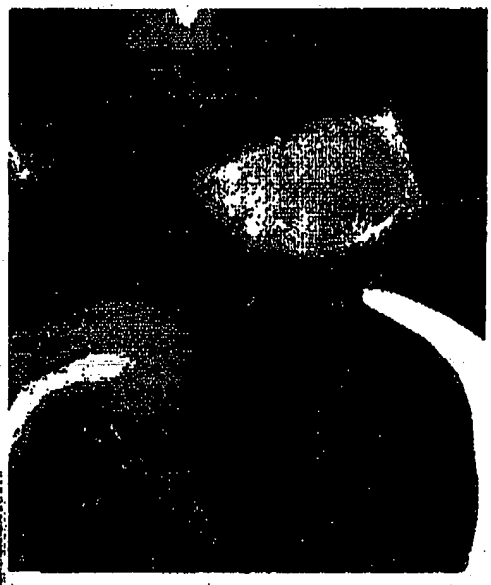

Figure 10.3. Macrophotograph showing white matter demyelination and necrosis secondary to carbon monoxide poisoning. Trichrome. (Original magnification ×2.)

Nitrous Oxide

Nitrous oxide has long been used as an anesthetic agent. Accidents with this and other inhalation anesthetics may lead to hypoxic hypoxia with pallidal necrosis and white matter lesions. These are morphologically quite similar to those seen after carbon monoxide intoxication. Recently, much attention has been focused on a myeloneuropathy encountered in individuals, most often dental personnel, who have had prolonged exposure to nitrous oxide. Although histopathologic studies in humans are lacking, the clinical manifestations are similar to subacute combined degeneration. The nitrous oxide is thought to produce its injurious effects by inhibiting vitamin B_{12} utilization and is especially prone to precipitate neurologic disease in individuals with subclinical B_{12} deficiency (92). Experimental studies with this agent have produced degeneration of the spinal cord involving both myelin sheaths and axons.

flective of recirculation, as glial swelling can only occur in the presence of some oxygen.

Respirator Brain

The designation "respirator brain" has been applied to the brain of patients who suffered severe global anoxic-ischemic episodes and were clinically comatose, without cephalic reflexes, usually with isoelectric electroencephalograms (EEGs), and with radiographic evidence of a severe reduction or absence of cerebral blood flow as a result of increased intracranial pressure. The brains are swollen, congested, dusky, and friable and remain soft despite adequate fixation (431, 691). There is usually transtentorial and tonsillar herniation with or without Duret hemorrhages. The ventricular lining sloughs off easily, and fragments of macerated cerebellum may occasionally be found alongside the spinal cord. The pituitary gland is often necrotic, and the sagittal and transverse venous sinuses may be clotted. It requires 12–24 hours after anoxia-ischemia to develop these changes (367, 486). The nature and duration of the offending process, extent of residual cerebral blood flow, and degree of acidosis influence the extent of neuropathologic change.

The histopathologic features, while variable, are similar to those of anoxia-ischemia, except for the absence of inflammation and lack of reactive glial or vascular changes. Neurons may show eosinophilic change, or the cytoplasm may be pale and ghost-like. Nuclear changes vary from extreme pallor to pyknosis. Glial cells show similar alterations. Blood vessels are often congested, and the endothelial cells show blurring of cytoplasmic detail and nuclear pallor.

The pathology of the "respirator brain" is believed to represent in vivo autolysis that was allowed to occur as a result of the patient being maintained on a mechanical respirator after an anoxic-ischemic episode. It should not be construed that the respirator in itself was in some fashion responsible for these pathologic changes (228, 486).

Postanoxic Encephalopathy

After an anoxic episode that is severe enough to produce coma, patients may recover in 24–48 hours, but after a period of 4–14 days they will occasionally develop confusion, disorientation, aphasia, and coma (246, 506). At autopsy, changes are confined to the white matter, although cerebral cortical and pallidal lesions have sometimes been described (Fig. 11.8). The mechanism for this delayed postanoxic state is unclear. The lesions are to a varying degree demyelinative and necrotic. Early lesions are in a perivascular location. The mechanisms involved appear to be a combination of prolonged but lesser degrees of hypoxia and depressed blood perfusion, perhaps with added hypocapnia (246, 439, 461). Kamijyo et al. (328) and Garcia and Conger (229) noted that with long-standing fluctuation in blood pressure, white matter necrosis became prominent. The reason for such sensitivity of the white matter to blood pressure fluctuations is not known. Okeda et al. (461) showed an unusual degree of vasodilation in white matter rather than in gray matter after anoxia, and Welsh et al. (704) noted a particular vulnerability of the white matter to energy failure in the setting of sustained perfusion failure.

Carbon Monoxide Intoxication

Carbon monoxide (CO) has 250 times the affinity of oxygen for hemoglobin, thus reducing the oxygen-carrying capacity of blood. When approximately two thirds of hemoglobin is converted to carboxyhemoglobin, death ensues. CO may also interact with cytochrome oxidase, perhaps adding a histotoxic component to the anoxia.

The pathologic changes in brain in acute lethal cases are minimal. The brain may appear pink-red as a result of the presence of carboxyhemoglobin. In addition, slight congestion and petechial hemorrhages may be evident. In longer surviving cases the changes are similar, if not

Figure 11.8. Postanoxic leukoencephalopathy. Coronal section of the brain shows symmetrical areas of necrosis in white matter. The presence of petechial hemorrhages within the necrotic zone is not usually found. (Courtesy of Myron D. Ginsberg, M.D.)

identical, to those already described for anoxia-ischemia. For unexplained reasons, the globus pallidus is commonly affected (Fig. 11.9), perhaps more so than in anoxia-ischemia, suggesting a specific cytotoxic effect of CO for the pallidum.

A delayed encephalopathy similar to that seen with anoxia-ischemia may occur (Grinker's myelinopathy). As in anoxia-ischemia, the hemispherical white matter bears the brunt of the pathologic changes resulting in symmetrical diffuse damage.

Symmetrical white matter lesions have been produced in monkeys after exposure to CO (250). The authors proposed that the combination of hypotension and metabolic acidosis led to white matter injury.

Hypoglycemia

The brain has a high metabolic rate and is very much dependent upon a constant supply of glucose for normal activity. The brain's respiratory quotient of 1 indicates that glucose is the predominant substrate in energy metabolism. Circulating glucose enters the brain via facilitated diffusion or saturable passive carrier-mediated mechanisms (52). Once glucose reaches the cellular compartment, it is metabolized almost exclusively by glycolytic and oxidative pathways (50). Cerebral stores of glucose and glycogen are sufficient to maintain an overall rate of energy consumption in humans for only a few minutes (51).

The brain may utilize exogenous substrates in certain circumstances such as starvation, when ketone bodies are generated. The use of ketone bodies as alternative sources of energy by the fasting adult may explain the occasional poor correlation between blood glucose levels and the severity of symptoms in hypoglycemia. On the other hand, neonates a

plantation (297, 681). The high incidence of lymphomas has usually been ascribed to the presence of a chronic antigenic stimulation provided by the transplant, which results in immunoblast proliferation with possible neoplastic transformation. The relatively high incidence of these tumors within brain has been attributed to the privileged immunologic conditions present in the brain, perhaps secondary to the lack of lymphatics and the presence of the blood-brain barrier, all of which would protect the neoplasm from an immunologic attack. In addition, the patient's capacity for immunologically responding to these tumors might be impaired by the associated immunosuppression.

CPM has been described in association with renal transplantation (579). This complication has only been observed in a minority of patients and might perhaps be more related to associated electrolyte disturbances than to the transplant itself (see above).

LIVER DISEASE

Hepatic Encephalopathy

Patients with liver disease may develop a neurologic syndrome commonly referred to as hepatic encephalopathy, hepatic coma, or portal-systemic encephalopathy. The clinical picture is characterized by a fall of intellect, personality changes, impairment in the level of consciousness, asterixis, and characteristic electroencephalographic changes. This syndrome may develop in a wide variety of liver disorders but is most commonly seen with alcoholic cirrhosis.

Etiology and Pathogenesis

The precise etiology and pathogenesis of hepatic encephalopathy are not known. The most favored view is that toxic substances elaborated by the gut are not detoxified by the liver, either because of the presence of shunts or because of inability of the injured liver to detoxify these substances. The principal issue, to date, has been which of the relatively large number of candidates may be the responsible factor. It is likely that several factors acting synergistically may give rise to the neurologic deficit, a concept strongly championed by Zieve (725).

Possibly the most important toxin and certainly the one that has received the greatest attention is ammonia. Strong arguments in support of a significant role for ammonia has been adequately marshaled by Conn and Lieberthal (127). They showed that although the correlation between blood ammonia and clinical symptoms has not always been perfect, it has been fairly good. Reasonable explanations have been offered by these authors to account for those occasional episodes where the correlation was poor. Further support for the etiologic role of ammonia is that any condition that results in an elevation of blood ammonia tends to aggravate hepatic encephalopathy (administration of ammonium salts and resins, hypokalemia, elevation of blood urea, gastrointestinal bleeding, and ingestion of a high protein diet) (140, 692). Importantly, almost all of the current therapeutic maneuvers currently employed in the therapy of hepatic coma are aimed toward the reduction of blood ammonia levels (87). Also, infants with congenital urea cycle defects that result in hyperammonemia have shown histopathologic changes similar to that seen in hepatic encephalopathy (102, 109, 371, 374, 406, 471, 619). Lastly, a wide variety of experimental situations that produce an elevation of brain or blood ammonia results in a histopathology identical with that seen in hepatic encephalopathy in humans (see Ref. 452 for review).

Ammonia is clearly neurotoxic (289). In animals, high doses result in seizures, while lower doses cause coma. It is still unclear how ammonia exerts its toxic effect in humans and animals. It was earlier suggested that ammonia may initiate a bioenergetic failure through the mechanism by which the brain detoxifies ammonia (73). Ammonia is detoxified by the formation of glutamine after the pathway

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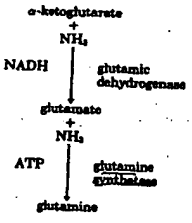

The detoxification of ammonia consumes ATP, NADH, and α-ketoglutarate, so that bioenergetic failure was a reasonable proposition. More recent studies by Hindfelt and Siesjo (292) and Hawkins and coworkers (273), however, have failed to identify bioenergetic failure at least in acute ammonia encephalopathy. Yet, the possibility that bioenergetic failure may be confined to a small but crucial compartment in brain has not been precluded. This is particularly important in that ammonia metabolism is a compartmentalized process (71) and the best evidence, to date, suggests that that compartment is the astrocyte (451, 457).

Ammonia has potent electrophysiologic effects. It depolarizes the cell membrane and causes hyperexcitability by blocking postsynaptic inhibition (392, 522). Additionally, ammonia may lead to abnormalities in acetylcholine metabolism (85, 479, 663). Ammonia may also affect the levels of GABA and glutamate (77, 290), substances that are putative neurotransmitters. A variety of other abnormalities induced by ammonia have been described, including effects on the activity of NA^+, K^+-ATPase (569), carbonic anhydrase (436), alterations in the blood-brain barrier (364), and loss of vascular autoregulation (342). (For review of these and other mechanisms, see Refs. 129 and 452.)

Other potential toxins have been proposed, but their evidence is not nearly as strong as for ammonia. Some of these include short chain fatty acids, α-ketoglutaramate, phenols, and mercaptans (728).

The false neurotransmitter hypothesis and its modifications has received considerable interest. It was originally proposed that gut-derived catecholamine by-products of bacterial decarboxylation (octopamine, phenylethanolamine, and perhaps others) are circulated around the liver and enter the brain to displace normal neurotransmitters. Serum levels of octopamine have appeared to correlate well with the level of encephalopathy (359, 398). The status of false neurotransmitters is currently uncertain, since the intraventricular injection of very high doses of octopamine to rats has so far been without clinical effect (730). Furthermore, the experimental reduction of norepinephrine and dopamine, substances that presumably are displaced by the false neurotransmitters, did not cause significant alterations in the level of consciousness in animals (406).

A modification of the false neurotransmitter hypothesis has been proposed by Soeters and Fischer (618). They observed a characteristic pattern in the amino acids found in plasma typified by elevation in the levels of aromatic amino acids (phenylalanine and tyrosine) and a depression in the levels of branched-chain amino acids (valine, leucine, isoleucine). The imbalance in the ratio of these amino acids leads to an increased entry of the aromatic amino acids into brain, resulting in an altered neurotransmitter status chiefly by raising serotonin levels. The elevation in brain glutamine (by ammonia) simply aids in the transport of these aromatic amino acids by acting as a coupling agent (309). Abnormalities in transport across the blood-brain barrier may also contribute to the alteration in the levels of neurotransmitters and their precursor amino acids (274).

Involvement of GABA and GABA receptors and of the related benzodiazepine system is currently being actively investigated (61, 566, 567). Increased numbers of GABA and benzodiazepine receptors have been found in animal models of hepatic encephalopathy. Similarities in clinical and electrophysiologic findings between hepatic encephalopathy and activation of GABA and benzodiazepine re-

ceptors have been made, and amelioration of symptoms has been reported with the use of benzodiazepine antagonists (434). However, not all investigators have been able to find abnormalities in these receptors or in GABA levels (106, 365, 394). It is unlikely that the GABA-benzodiazepine hypothesis as originally proposed is correct (565). The possibility of an endogenous benzodiazepine ligand has recently been proposed to explain the response to benzodiazepine antagonists (434, 462).

Pathology

The only consistent histopathologic change observed in patients dying from portal-systemic encephalopathy has been a change in gray matter astrocytes referred to as the Alzheimer type II changes (protoplasmic astrocytosis). Although the association of astroglial changes to acquired liver disease was made earlier by Scherer (570), it was the classical and detailed studies of Adams and Foley (6) that clearly emphasized the characteristic astroglial changes associated with liver disease.

The Alzheimer type II astrocyte as observed in hematoxylin-eosin (H&E) preparations is characterized by an enlarged, vacuolated nucleus with chromatin margination and often a prominent nucleolus (Fig. 11.14). The astrocytes show little visible cytoplasm, which frequently contains excessive lipofuscin pigment. Intranuclear inclusions consisting of glycogen may be observed particularly in chronic cases (679) (Fig. 11.14*C*), and occasionally some glycogen may also be found in the cytoplasm (305). Diminished amounts of glial fibrillary acidic protein (GFAP) have recently been emphasized (340, 617).

These astroglial changes are found throughout the gray matter of brain and are only minimally observed or even absent in the white matter. In the cerebral cortex they are best noted in the deeper layers. They are also prominent in the striatum, globus pallidus, thalamus, substantia nigra, inferior olives, dentate nucleus, and the Bergmann cells of the cerebellum. The brain stem and spinal cord show a lesser degree of this change. Alzheimer type II cells are not observed in the pyramidal layer of the hippocampus, yet may be conspicuous in the adjacent subiculum. The reasons for these topographical differences are not known. For unexplained reasons, Alzheimer type II cells tend not to be conspicuous in patients with hepatorenal syndrome (Fig. 11.14*D*).

In the cerebral cortex, striatum, and thalamus, the astrocyte has a rounded outline. However, in the pallidum, substantia nigra, inferior olives, and dentate nucleus of the cerebellum, the astrocyte is highly lobulated (Fig. 11.15). Whether this morphologic variation represents distinct and separate responses to liver injury or whether it merely reflects local anatomical differences is not known. It is likely that the latter view is correct and that there is probably no significant differences between the rounded "naked glial nuclei" as observed in cortex and the more lobulated forms as originally described in the pallidum by Von Hoeslin and Alzheimer (687).

Another characteristic astrocyte response to liver disease is the apparent increase in the number of these cells. Typically, astroglial nuclei are clustered in pairs, triplets, and even quadruplets (Fig. 11.14*B*). Adams and Foley (6) indicated an approximate twofold increase in these cells. The conspicuous lack of mitotic figures, however, was and continues to be a puzzling aspect of this astroglial response. A study by Brun et al. (101) also showed an increase in the number of astrocytes, but they observed no increase in the total number of glial cells.

Experimental studies concerned with the issues of astroglial proliferation have been performed. Norenberg (450) and Taylor et al. (648) noted an apparent proliferation of astroglial cells in their models of hepatic encephalopathy. Again, mitotic figures were not observed. Diemer (15[illegible] has extensively studied the problem of quantitation and noted an increased number of Alzheimer type II cells. Similar to the observations of Brun et al. (10[illegible]

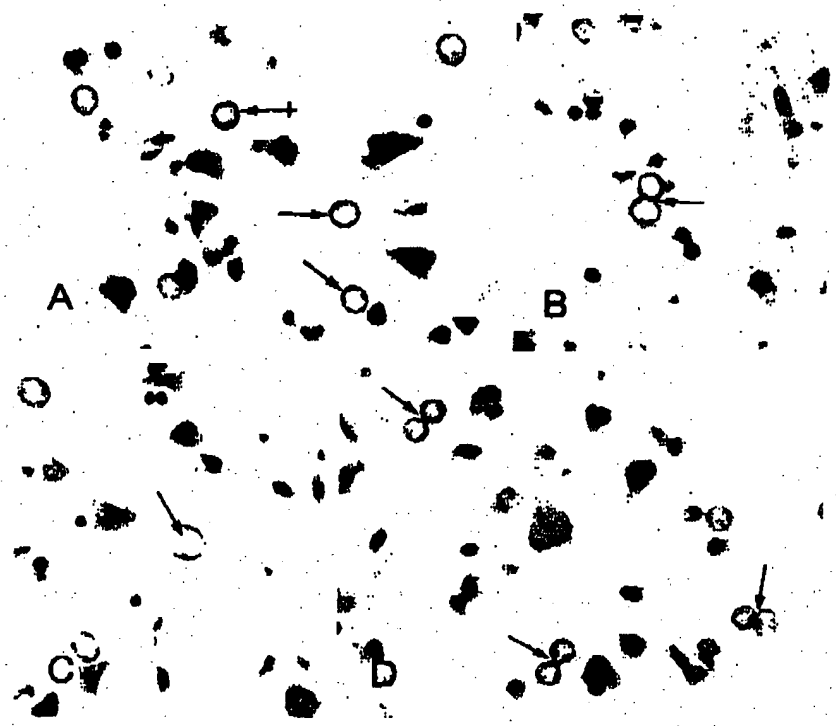

Figure 11.14. *A*, Alzheimer type II astrocytes from a patient with hepatic encephalopathy are characterized by enlarged vacuolated nuclei with margination of chromatin *(arrows)*. A comparably less involved astrocyte is indicated by *cross-arrow*. *B*, Astroglial hyperplasia is manifested by the presence of paired nuclei *(arrow)*. *C*, PAS-positive intranuclear inclusion in an Alzheimer II astrocyte *(arrow)*. *D*, Section from cerebral cortex of a patient with hepatorenal syndrome. Note that the characteristic vacuolization of Alzheimer type II cells is not fully developed, although astroglial proliferation is represented by the presence of three paired nuclear forms *(arrows)*. (From Norenberg MD: The astrocyte in liver disease. In *Advances in Cell Neurobiology*, edited by Federoff S, Hertz L, vol 2, p 321, fig 4, New York, Academic Press, 1981.)

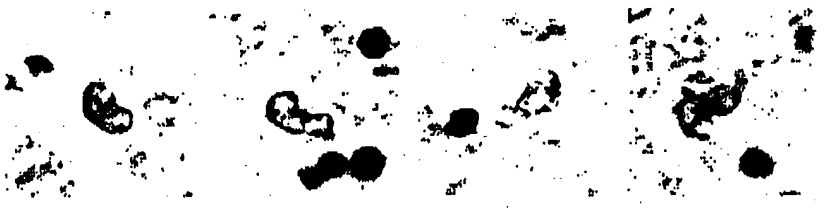

Figure 11.15. Representative forms of Alzheimer type II astrocytes from globus pallidus in hepatic encephalopathy showing varying degrees of nuclear lobulation and irregularity. H&E. Original magnification ×600.) (From Norenberg MD: The astrocyte in liver disease. In *Advances in Cell Neurobiology*, edited by Federoff S, Hertz L, vol 2, p 322, fig 6, New York, Academic Press, 1981.)

in humans, Diemer was not able to find an increase in the total number of glial cells. He concluded that a true proliferation of astrocytes in hepatic encephalopathy probably does not occur but rather that cells not originally recognized as astrocytes are subsequently interpreted as astrocytes once they developed the typical Alzheimer type II appearance. A recent study in hyperammonemic animals by Brumback and Lapham (100) has shown that astrocytes incorporate thymidine, although mitotic figures were not observed. In any event, while the issue of glial proliferation remains controversial, the apparent proliferation is indeed one of the most characteristic features of the histopathology of human hepatic encephalopathy.

Electron microscopic studies of hepatic encephalopathy in humans have been few. Martinez (407) observed that the astrocyte cytoplasm was swollen and contained membrane-bound vacuoles. Minor mitochondrial changes and an apparent increase in the liopfuscin pigment were observed. Foncin and Nicolaidis (212) demonstrated enlarged, irregular nuclei with coarse chromatin. The cytoplasm was increased in size and was associated with an increase in ribosomes and glycogen.

There is a fairly good correlation between the clinical severity of hepatic encephalopathy and the extent of these astroglial changes (6, 452). Since hepatic encephalopathy may be clinically reversible, one suspects that these astroglial changes may also be reversible. However, a definitive statement on this aspect in humans cannot be made at this time. This issue has been studied experimentally by Diemer and Laursen (160) and by Norenberg (452) who have shown that it is a reversible process at least in experimental settings.

Experimental Studies

A large number of experimental studies have been performed to assess the astroglial response to liver disease. These have been summarized (452, 454). Most of these studies were designed either to injure the liver or to elevate the blood and/or brain ammonia content. With a few exceptions, all studies have resulted in the formation of cells resembling the Alzheimer type II change seen in humans.

Electron microscopic studies by Zamora et al. (725), Norenberg and Lapham (456), and Norenberg (450) showed similar findings. These consisted of hypertrophic cytoplasmic changes including a proliferation of mitochondria and RER (Fig. 11.16). These changes occurred early in the phase of this disorder. As the clinical state worsened, the cytoplasmic features appeared degenerative and were characterized by hydropic degeneration, presence of membrane-bound vacuoles, degenerated mitochondria, and swollen Golgi and endoplasmic reticulum (Fig. 11.17). These degenerative changes occurring terminally were the electron microscopic counterpart of the Alzheimer type II cell as seen via light microscopy. Significant nuclear alterations were not observed. This is in keeping with the observations of Cavanagh and Kyu (112) and Norenberg and Lapham (456) that the nuclear swelling observed by light microscopy is, in part, an artifactual change.

It was tentatively concluded that the initial hypertrophic astroglial appearance reflected heightened metabolic activity perhaps for ammonia detoxification. This appears plausible in that glutamine synthetase, the principal enzyme involved in ammonia detoxification, is exclusively found in astrocytes (457). Eventually, perhaps because of energy failure, degenerative changes develop. Thus, impaired astroglial function (water, electrolyte, pH, neurotransmitter regulation) (see Ref. 584 for review) may lead to an encephalopathic state.

Fulminant Hepatic Failure

After acute toxic or viral injuries to the liver, patients may develop an explosive syndrome characterized by the rapid development of delirium, coma, and seizures. Alzheimer type II changes tend not to be very striking, although they are present. Perhaps the short duration of the

GREENFIELD'S NEUROPATHOLOGY

FIFTH EDITION

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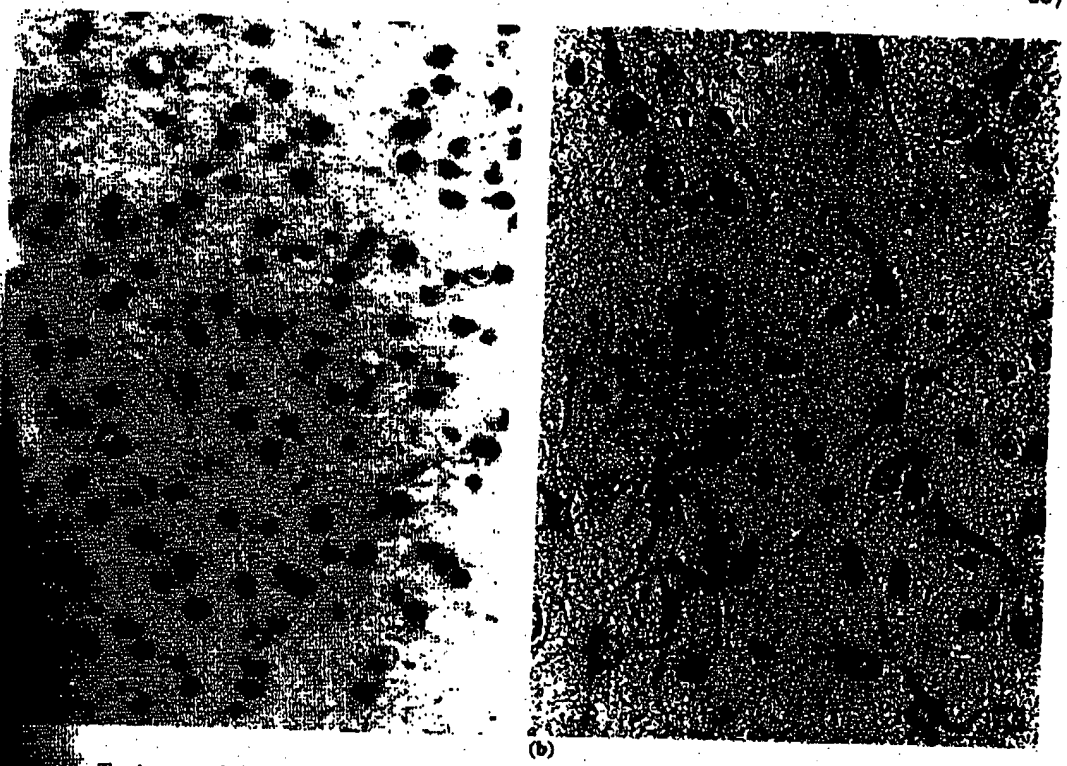

(b)

Total neuronal destruction with proliferation of protoplasmic astrocytes in the thalamus of a 7-month-old born infant. Haematoxylin and eosin. (b) Immunoperoxidase stain for glial fibrillary acid protein of the thalamus of with perinatal asphyxia. Note replacement of neuronal population by reactive astrocytes, many of which have a

amage to white matter. Specifically e PVLM, focal or general gliosis of , necrosis of individual glial cells, ation of amphophilic globules under Then, in 1978, Takashima, nd Becker added yet another diag- , subcortical leucomalacia, to sep- in which necrosis was limited to below the central cortex. terms precisely describes the attempted to define but they ition, as they are established erature. The controversial sub- change of the white matter ed, as it is most likely not a gn. An excellent discussion und in Friede's text (1989).

PERIVENTRICULAR LEUCOMALACIA

Predilection of cerebral white matter in the infant brain to undergo necrosis has been known for over 120 years. Since Parrot (1868) originally called attention to this phenomenon, others have added descriptions of the morphological features and proposed a number of pathogenetic events to account for its presence.

Although tissue changes have been well described, there exists no consensus as to aetiology. Review of the literature by Rorke (1982), Larroche (1984) and Gilles (1985) yields a list of factors thought to be important in pathogenesis. The significance of several is questionable, as, for example, inanition, originally reported by Parrot

Fig. 11.19 Typical Alzheimer type 2 astrocyte in a deep grey nucleus of a 3-day-old infant. Haematoxylin and eosin.

(1868), or icterus, as emphasized by Schmorl (1903). Nor has there been much attention by others to the findings of Leviton and Gilles (1984) of a relationship between white matter necrosis and congenital visceral anomalies, 'unfavourable intrauterine environment' and certain adverse socioeconomic factors of the mothers. Earlier writers suggested that thromboembolic phenomena played a role in the pathogenesis of these lesions (Gilles, 1985), but additional data which might lend support to this notion have not been forthcoming.

Two other important possible causes of white matter necrosis are infections and circulatory disturbances. In fact, the effect of the former may be mediated through the latter. Gilles (1985) has been the major protagonist for an infectious origin of white matter damage and cites support for his view from studies by Herschfeld, Perrin and Landing and Gluszcz, as well as his own experimental and epidemiological studies (Leviton and Gilles, 1983, 1984). Whereas Glusz (1961) thought that sepsis of bacterial, protoz or even fungal origin might be responsib Gilles, Shankle and Dooling (1983), Morishi Niemann and James (1978) and Young et (1981) have focused on bacterial endotoxins.

Bejar et al. (1988) diagnosed white matter n rosis in 23 of 127 infants, the onset of which considered to be antenatal in 13. Aside from inverse relationship of incidence to weight, t investigators noted a significant direct associa with placental vascular anastomoses in mu pregnancies, funisitis and purulent amn fluid.

Studies by Morishima, Niemann and (1978) and Young, Hernandez and Yagel (suggest that the effect of the endotoxaemia be mediated through circulatory collaps acidosis or, alternatively, by vascular d resulting in breakdown of the blood–brai rier (Veith, 1961).

The prevailing opinion regarding the genesis of white matter necrosis in the brain ascribes these to circulatory derange Rydberg (1932) was an early proponent idea and there has been a growing body dence since then in support of this conc the same time, there are conflicting data ing specific details. In their now-class Banker and Larroche (1962) emphasized in terminal arterial territories; later, (1984) cited studies of van den Bergh (1 den Bergh and Vander Eecken (1968) Reuk, Chatta and Richardson (1972), a were of the opinion that the white ma occurred at the boundary between fugal (long medullary penetrating ar ventriculopetal (recurrent collaterals ventricular) arteries.

Gilles (1985) challenged this conc basis of studies done with Kuban Gilles, 1985), and cited three objec cept for the striatal arteries, vascula the telencephalon are not musculor called 'recurrent collaterals' and trans arteries are rare or non-existent in forebrain during the last half of gesta primary localization of lesions in frontal and occipital poles with central brain regions is difficult lesions truly localize in border zo

wever, Gilles (Gilles and Leviton, 1984) a contradictory statement: 'Available evi- indicates that this disorder (or group of ders), named acquired perinatal leukoen- alopathy, reflects a decrease in blood flow ough the distal vessels supplying para- icular white matter . . .' and cites micro- raphic studies done by Takashima and (1978), who reported the existence of ntriculofugal and ventriculopetal vessels, alization of softening between and at the of these medullary arteries.

se conflicting observations notwithstand- is likely that the nature of the vascular is not the sole pathogenetic factor. s, in fact, mounting evidence that hypo- of white matter during severe hypoten- ow cerebral blood flow (<10 ml/100 g per as suggested by Lou and others (Lou, and Friis-Hansen, 1979; Lou, 1980, ung, Hernandez and Yagel, 1982; Lipp- et al., 1989), and certain metabolic produced by hypoxia may also be of hogenetic significance.

of puppies by Kennedy and co- 1970, 1979) disclosed a higher meta- of white matter early in development after maturation is completed. There able overlap in rates of blood flow to hite matter for a few days after birth pears at about 1 month of age, when matter is clearly greater than it is to (Kennedy et al., 1972).

nd Duffy (1979) observed increased olism in white matter during hypo- an increase in anaerobic glycolysis relative to grey matter. However, in normal and hypoxic dogs by Duffy (1982) showed no compen- ia in white matter during hypo- ing these tissues exquisitely jury. It has also been observed e of glycolysis in white matter exceeds the substrate supply 82), leading to a switch from ic metabolism followed by a acid and oxygen-derived free legenstein and Winegar, 1984; ten-Myers, 1985).

Clinical features of periventricular leucomalacia

Prior to the availability of non-invasive radiological techniques, the diagnosis of PVLM could only be made *post mortem*, as there are no reliable clinical correlates of the lesion in the neonatal period (Volpe, 1987). However, ultrasonographic evaluation is currently common and its use has allowed clinical diagnosis of this lesion in the cystic stage (Bozynski et al., 1985) If CT scanning is done in the newborn period, care must be taken not to confuse periventricular hypodensities secondary to absence of myelin with necrosis, particularly in preterm infants (Quencer, Parker and Hinkle, 1980). Chronic lesions are seen without difficulty using MRI (Lipper et al., 1988).

Infants who survive often have developmental delay of varying degrees, severe motor dysfunction (i.e. quadriplegia or diplegia) and, often, impaired vision (Weindling et al., 1985; Volpe, 1987).

The frequency of PVLM varies according to the population from which material is drawn. An incidence of almost 7 per cent was observed among an unselected group of 212 neonates weighing less than 2500 g examined *post mortem* at the Philadelphia General Hospital (Rorke, 1982). This figure is considerably higher if a low birth weight, high-risk population is evaluated; Larroche (1982) identified lesions of this type in 35 per cent of such a group.

Risk factors associated with white matter necrosis include serious disturbance of cardiorespiratory function associated with respiratory distress syndrome, congenital cardiac malformations, shock or sepsis. Larroche (1977a) reported an association with intrauterine growth retardation, possibly related to hypoglycaemia, and postmature infants as well as those born of diabetic mothers also appear to be at risk for white matter necrosis. Although the lesion is slightly more common in infants weighing less than 2500 g, it is by no means rare in the term infant (56 per cent vs 44 per cent) (Rorke, 1982).

White matter necrosis may also occur *in utero*, most commonly in association with disturbance in maternal–fetal circulation and sepsis (Terplan, 1967; Larroche, 1986; Nakamura et al., 1986; Bejar et al., 1988) or without a definable cause (Ellis, Goetzman and Lindenberg, 1988).

Pathology of periventricular leucomalacia

Acute necrosis of white matter (Figs. 11.20 and 11.21) may appear anywhere as poorly defined areas of liquefaction or cavitation, with or without haemorrhage or as white or yellow-white spots, measuring about 3–6 mm in diameter, but is most commonly adjacent to the lateral ventricles. Tissue destruction often extends from the ventricle for a variable distance into the centrum ovale and occasionally reaches the subcortical white matter. Focal lesions most commonly occur in white matter around the anterior and posterior horns of the lateral ventricles, but they are also found adjacent to the body of the ventricles.

Acute lesions are characterized microscopically (Fig. 11.22) as areas of coagulative necrosis which are either less or more brightly stained than adjacent intact tissues. Reactive microglial cells usually appear within 8 hours, and reactive astrocytes and capillary endothelial proliferation become obvious around 12 hours after the ictus (Banker and Larroche, 1962). Polymorphonuclear leucocytes and lymphocytes are conspicuous by their absence (Armstrong and Norman, 1974), but there is often a haemorrhagic component to the lesion. Retraction balls are recognizable within 24–48 hours (Figs. 11.23 and 11.24) but more extensive axonal changes are not well seen unless special silver impregnation techniques are used. Deposits of basophilic or meta-

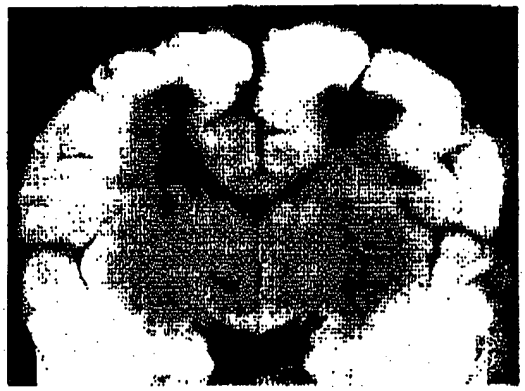

Fig. 11.21 Acute white matter necrosis with a haemorrhagic component in the brain of a premature infant. Note the extension of the lesion from the periventricular zone to subcortical white matter.

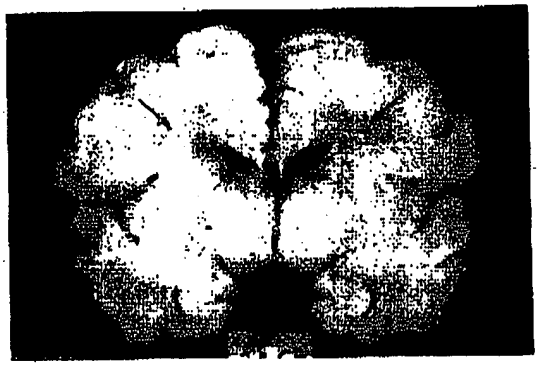

Fig. 11.20 Periventricular leucomalacia. Coronal section of the brain of a premature infant. White spots, each several millimeters in diameter, are shown (arrows) close to the lateral ventricles.

Fig. 11.22 Acute coagulation necrosis in periventricular white matter of a term infant with congenital heart disease. Note the bland character of the lesion and absence of cellular reaction. Haematoxylin and eosin.

11.23 Focus of white matter necrosis containing retraction balls, lipid-laden macrophages and astrocytes. Haematoxylin and eosin.

Fig. 11.24 Partially organized foci of necrosis and haemorrhage in white matter in the brain of a 15-day-old infant. Note the presence of numerous retraction balls, a small focus of necrosis (arrow) and a well demarcated haemorrhage. Haematoxylin and eosin.

matic granular or irregular stick-like cial are sometimes found in damaged tissues. 2 weeks there is clear demarcation of the and prominent macrophage activity such e lesion consists of a mixture of red cells (or siderin), lipid-laden macrophages, reacrocytes and newly formed vessels.

chronic phase the damaged white matter puckered, retracted or frankly cystic 1.25), the cysts being uni- or multilocu- The ventricles, at the level of damage, are dilated and the corpus callosum is often in width.

copic features vary according to the exissue destruction. In non-cystic foci, ietaris is often encrusted with calcium salts. Hypertrophic astrocytes, lipidcrophages and cholesterol clefts are d haemosiderin deposits may also be chow–Robin spaces of neighbouring

Fig. 11.25 Bilaterally symmetrical multicystic lesions in a 7-month-old prematurely born infant with severe respiratory distress syndrome. Note the thin corpus callosum and ventricular dilatation.

blood vessels are often filled with lipid-laden macrophages.

The larger cystic lesions are basically similar except for the presence of a cavity or cavities. Reactive cellular changes are found in the wall and extend for a variable distance into surrounding tissue.

It is unlikely that the rare cases of familial porencephalic white matter disease (Berg, Aleck and Kaplan, 1983; Smit et al., 1984) are pathogenetically related to this perinatal problem, but descriptions by Benda (1945) and Schwartz (1961) of cystic degeneration of white matter and the 'bladder-like' central porencephalies probably do represent the terminal evolution of widespread *in utero* or perinatal white matter necrosis.

PERINATAL TELENCEPHALIC LEUCOENCEPHALOPATHY

This term was introduced by Gilles and Murphy (1969) to describe damage to telencephalic white matter during the perinatal period, and included necrosis and hypertrophy of glial cells, deposition of amphophilic globules and foci of necrosis anywhere. In fact, 18 per cent of the 271 brains included in their study group had PVLM. Whilst their findings suggest a continuum of damage to developing white matter, the nomenclature introduced unnecessary confusion. Since the majority of brains (87 per cent) displayed glial necrosis and/or hypertrophy, a separate category for this type of lesion might have been more useful than an umbrella term that included a previously described entity. The major objection, however, is to the use of the term 'telencephalic', as white matter anywhere in the nervous system may undergo damage.

Although little attention has been focused upon white matter gliosis, it has nevertheless been well described in the cerebellum and spinal cord, as well as in the cerebrum (Leech and Alvord, 1974; Rorke, 1982). In the writer's experience, however, amphophilic globules and necrosis of glial cells are not so prominent as Gilles and Murphy suggest.

White matter gliosis is found with equal frequency in preterm and term infants, may be well developed in the newborn brain and is not necessarily associated with any specific intrauterine problem, although Gilles and Murphy (1969) found a slightly greater frequency of perinatal difficulty among their cases. Hypoxia–ischaemia probably plays a role in pathogenesis, as about 60 per cent of infants with white matter gliosis have respiratory distress syndrome, congenital heart disease or perinatal asphyxia, and it is sometimes striking in postmature infants (Rorke, 1982).

Astrocytic proliferation in white matter is often coupled with retardation in myelination in association with nutritional deficiency (Dickerson, Dobbing and McCance, 1966; Bass, Netsky and Young, 1970; Winick, 1976), congenital rubella syndrome (Rorke, 1973) or in bronchopulmonary dysplasia (Takashima and Becker, 1984). Incidence varies from 15 per cent (Rorke, 1982) to 40 per cent (Gilles and Murphy, 1969).

White matter gliosis alone is unassociated with any specific clinical signs, although widespread involvement, particularly when accompanied with retardation in myelination would be expected to result in psychomotor retardation of some type.

The diagnosis cannot be made by gross examination; white matter may be swollen in the acute phase or diminished in volume at a chronic phase thus leading to mild hydrocephalus *ex vacuo*.

The condition is readily diagnosed histologically. There may be diffuse pallor of the affected tissue, examination at higher magnification often shows a microcystic or rarefied character and tissue may appear hypocellular (Fig. 11.26). Gemistocytic fibre-forming astrocytes (Fig. 11.27) often considerably overshadow the oligodendrocytes or myelination glia that may remain. Microglia as rod forms or lipid-laden macrophages may also be present, and amphophilic globules and karyorrhectic glia inconsistently found.

NON-PERIVENTRICULAR WHITE MATTER NECROSIS

The third category of white matter lesion described in the immature brain is subcortical comalacia which Takashima, Armstrong and Becker (1978) proposed is related to the development of blood vessels in cerebral sulci. Banker and Levene (1985) observed three infants with lesions in this site, and Clapp et al. (1985) produced subcortical white matter necrosis in rats by intermittent partial occlusion of the ma

.26 White matter damage in a term infant with ital heart disease characterized by loss of normal glia ltration by large lipid-laden macrophages. Haema- and eosin.

Fig. 11.27 White matter gliosis characterized by the spongy character of neuropil and preponderance of gemistocytic fibre-forming astrocytes with intracytoplasmic 'inclusions'. Immunoperoxidase stain for glial fibrillary acidic protein.

ion for 1 minute of every 3 minutes for 2

s of this type are not confined to depths l cortical sulci but are found, as well, in l white matter of the cerebellum (Fig. d see figures 49 and 50 in Rorke, 1982). ological features are similar in all re- focal ischaemic or haemorrhagic nec- more mature brain.

iciently emphasized in the literature is complete or incomplete white matter volving the cerebrum, the cerebellum though it is more common in the Rorke, 1982; Paneth et al., 1990). damage is often seen in association ion of the vein of Galen, in which is extensive secondary calcifica- man and Becker, 1974; Phillips, Dooley and Camfield, 1986). Selective necrosis of fibre tracts in the brain stem and spinal cord has not been observed, but the corpus callosum may be the sole target of injury.

Gross features in the acute–subacute phase may consist of a sunken translucent white matter, or tissue may have a softened granular character.

At microscopical level there is a patchy pattern of staining, breakdown of normal architecture and a brisk macrophage reaction. Small vessels within the lesion are generally engorged and endothelial cells are swollen. Large areas of damage often progress to cyst formation and, if multiple, lead to a condition called multicystic encephalopathy (Fig. 11.29), cystic sclerosis or the bladder-like porencephalies referred to above. Less complete destruction is characterized by a vacuolated appearance of white matter

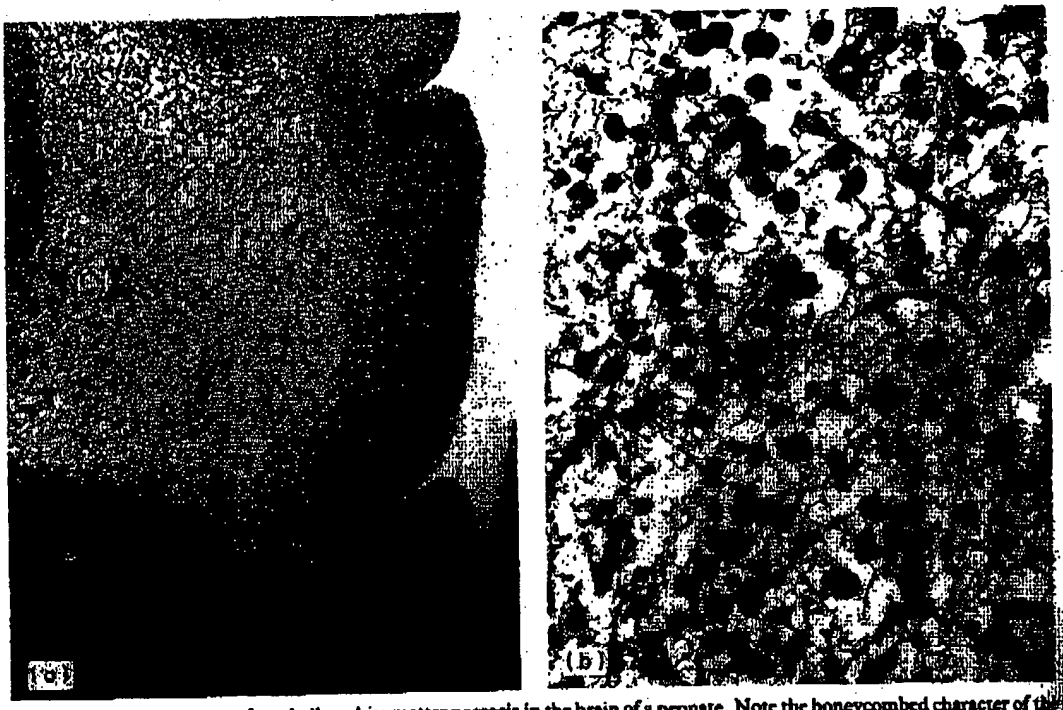

Fig. 11.28 (a) Large area of cerebellar white matter necrosis in the brain of a neonate. Note the honeycombed character of the lesion. Haematoxylin and eosin. (b) Higher magnification of necrotic area, showing a preponderance of lipid-laden macrophages. Haematoxylin and eosin.

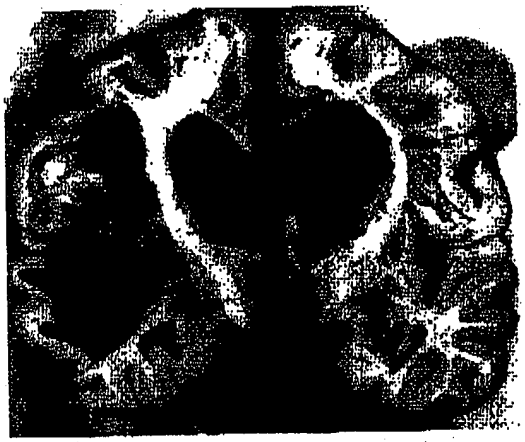

Fig. 11.29 Multicystic encephalopathy (cystic encephalomalacia) of subcortical white matter. There is also bilateral softening of the putamina.

throughout which macrophages and astrocyt... are scattered (Fig. 11.30) (for details see Rork... 1982).

Lesions of the corpus callosum may occu... association with white matter damage elsewh... but in rare instances is the sole site of lesions... an earlier description of four infants with cal... injury it was suggested that such isolated le... were probably clinically silent (Rorke, 1... Postmortem studies of several infants with... infantile myoclonic epilepsy have disclosed... spread necrosis of the corpus callosum... secondary atrophy of centrum ovale and mi... cephaly unassociated with significant co... lesions (1990, unpublished observation).

GREY MATTER LESIONS

Clinicians tend to make a diagnosis of ... ischaemic encephalopathy (HIE) in in...

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Oligodendrocyte/myelin injury and repair as a function of the central nervous system environment

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Abstract

Multiple sclerosis is a chronic neurologic disorder considered to result from relatively selective immune mediated injury of central nervous system (CNS) myelin and/or its cell of origin, the oligodendrocyte (OGC). Constituents of both the innate and adaptive immune systems are potential contributors to this process. Endogenous (microglia) and infiltrating (macrophages, dendritic cells) constituents of the innate immune system serve as sensors of events occurring within the CNS; their response to such events underlies the extent of their interaction (chemoattraction, antigen presentation) with the components of the adaptive immune system (αβ T cells, B cells) and ultimately the extent of the resultant inflammatory response. Constituents of both the innate and adaptive immune system can serve as effectors of tissue injury. The susceptibility of specific types of neural cells to injury further reflects the extent to which immune mediators modulate expression of crucial molecules (adhesion molecules, receptors) involved in effector–target interactions. Ongoing interactions between the constituents of the immune system themselves and between these constituents and neural cells are important determinants of disease recurrence and/or progression. Conversely, these interactions also impact on the mechanisms involved in target protection and repair.

Keywords: Multiple sclerosis; Microglia; Innate immunity; Oligodendrocytes/myelin; Demyelination/remyelination

1. Introduction

Multiple sclerosis (MS) is a neurologic disorder that characteristically evolves its course over many years. The most usual initial clinical manifestation is an acute or subacute neurologic deficit reflecting dysfunction within the CNS, followed over subsequent days or weeks by substantial recovery. Both the initial and subsequent exacerbations correlate with times of immune activation, usually exposure to infections. The magnetic resonance imaging (MRI) defined correlate of such events is a new lesion that may enhance if gadolinium has been administered systemically. The pathologic correlate of the lesion is a T cell dominated inflammatory perivascular response with both lymphocytes and macrophages extending into the parenchyma. Within the tissue, there is destruction of myelin with relative but not absolute sparing of axons. New lesions resolve to variable extents but may reappear including with enhancement during subsequent relapses. Serial MR studies using specialized techniques such as MR spectroscopy or magnetization transfer imaging (MTR) indicate the involvement of the "normal appearing" white matter (NAWM) even in the early phase of disease and that subsequent new lesions are biased to occur where such NAWM changes are most apparent [1]. In the later phases of the disease, the neurologic deficit appears to accumulate progressively even in absence of clear-cut relapses. The chronic active MS lesion, characteristic of the later disease phase features macrophage dominated tissue destruction with loss of both myelin and oligodendrocytes (OGCs) and axons, associated with a prominent gliotic reaction. These pathologic features underlie the postulate that immune mediated mechanisms underlie the development of the disease. Currently, there are no animal models of a similar disease occurring spontaneously in out-bred animals raised in "dirty" environments. This report focuses on the interactions between the constituents of the immune system and the CNS that can contribute to the tissue injury and repair that characterize MS, and how these interactions are themselves modulated by the CNS environment.

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2. Initiation of the MS disease process

The currently favored postulate is that MS is triggered by immune responses directed against myelin or its cell of origin, the OGC. Observations from both clinical disorders and experimental models indicate that the initial events leading to immune mediated CNS demyelination can occur either within the systemic compartment or within the CNS. The syndrome of post vaccination encephalomyelitis (or ADEM) that can complicate immunization with vaccines containing neural tissue illustrates the former sequence of events and can be re-produced in animals by systemic administration of CNS tissue, crude white matter or myelin, or purified myelin antigens or peptides. The animal disorder is referred to as experimental autoimmune encephalomyelitis (EAE). Speculation remains that exposure to virus contained peptide sequences that have homology to myelin components, an occurrence referred to as molecular mimicry [2], may trigger the autoimmune response in MS. Prototypes of immune mediated demyelinating disorders initiated by events within the CNS compartment are provided by the experimental chronic neurologic disorders arising in mice consequent to intra-cerebral infection with corona and Theiler murine encephalomyelitis viruses. Disease relevant autoreactive T cells develop as result of antigen release consequent to the initial viral induced tissue injury [3]. Such T cell sensitization could occur either within the CNS or in regional lymph nodes since antigens released within the CNS can be transported from the CNS to regional lymph nodes.

The extent of inflammation in the active MS lesions indicates that there must be substantial recruitment of systemic immune cells, lymphocytes and monocytes, from the systemic compartment into the CNS. Such recruitment would require that the cells cross the BBB and enter an environment that would promote their persistence and function. The actual transmigration across microvessels is dependent on active molecular interactions involving chemoattraction and adhesion. Products released by microglia and astrocyte, both of which extend foot process to the microvessels, can modulate the permeability properties of the blood brain barrier [4]. Animal based studies indicate that T cells accessing the CNS from the systemic compartment will persist in the CNS only if presented with the antigen they can recognize by competent antigen presenting cells (APCs).

The process of competent antigen presentation requires two signals, namely antigen presentation to the T cells receptors of antigen in context of major histocompatibility molecules (MHC) (class II for CD4 T cells; class I for CD8 T cells) and a second set of molecules referred to as co-stimulatory molecules, prominent amongst which are CD80/86 that interacts with CD28/CTLA-4 and CD40 that interacts with CD154. The perivascular microglia are the cell type that in situ most prominently expresses both MHC and co-stimulatory molecules and in vitro are highly competent antigen presenting cells [5]. Dendritic cells are likely also present in the perivascular space. Within the CNS parenchyma, the microglia can express MHC class II and co-stimulatory molecules [6]. Microglia are constituents of the innate immune system and as such serve as sensors for "stranger" (derived from exogenous agents such as viruses or bacteria) or "danger" (derived from injured or dying tissue) signals in the environment [7]. In context of MS, the microglia also interact with invading immune cells and their products. The above signals can amplify or suppress the state of activation of the microglia, and thus be important determinants of whether microglia will or will not promote a persistent neuroinflammatory response. The invading immune response in MS also results in entry into the CNS of innate immune cells (monocytes/macrophages, dendritic cells) that can participate in the process of antigen presentation.

3. Basis of selective OGC/myelin injury in MS

In this section, we will consider how the relative selectivity of OGC/myelin injury could be conferred either by the properties of the immune effector cells or molecules that are present in the active lesions or by those of the targets and how these properties may be influenced by the CNS environment.

4. Effector determined selective tissue injury

The antigen receptors expressed by constituents of the adaptive immune system, namely immunoglobulin (Ig) itself for antibody producing cells (B cell lineage) and $\alpha\beta$ receptor chains ($\alpha\beta$ TcR) for T cells have a high degree of diversity, consequent to the process of rearrangement of the genes that contribute to their structure. This diversity would allow for recognition of a vast array of target specific determinants.

5. Antibody mediated immune responses

The Ig deposited in the lesions of MS patients and recovered from the cerebrospinal fluid (CSF) includes antibodies that react with myelin components. These can be directed at protein, carbohydrate or lipid moieties. In vitro studies have not yet, however, shown that serum or CSF from MS patients selectively and specifically induces injury of myelin producing cells even in presence of complement (reviewed in [8]). Myelin/OGC antibody could also contribute to selective tissue injury by directing cells of the innate immune system present in the inflammatory environment of MS lesions to specific targets. Members of the innate immune system including $\gamma\delta$ T cells, NK cells and microglia/macrophages can all effect tissue injury by release of an array of mediators but due to their limited receptor heterogeneity, would not be expected to recognize targets with the specificity of the adaptive immune system constituents. These cells all express Fc receptors that can bind with the Fc portion of Ig molecules that are bound via their variable regions to specific targets.

adding to the complexity of using specific antigen or T cell receptor directed therapies.

10. Progression of the disease process

The pathologic features of the more chronic MS lesions include extensive destruction of OGCs/myelin associated with a microglia/macrophage rather than lymphocyte dominated immune response. Such pathology can reflect repeated immune mediated injury of both OGCs/myelin and axons. As previously mentioned, we found that oligodendrocytes made to over-express p53 are more susceptible to TRAIL and fas ligand mediated injury [14]. We further found that p53 is over-expressed in oligodendrocytes in MS lesions that feature prominent OGC cell death. P53 can be up-regulated by an array of insults including ischemia, infection, and trauma. We postulate that such up-regulation may reflect initial sublethal injury of these cells making them very susceptible to subsequent insults that can lead to lethal injury. The chronic MS lesion also features extensive axonal loss that could reflect either direct immune mediated injury or the consequences of the loss of myelin on the trophic factor requirements and physiologic properties of axons [24].

11. Consequences of systemic immunotherapies in the neurobiologic aspects of the MS disease process

Current therapies for MS are all administered systemically and are aimed at interrupting immune mediators involved in mediating the disease process. Therapies are most effective in the early disease phase that features frequent inflammatory lesion formation and ineffective in the late progressive disease phase. Interferons up-regulate expression of many genes including those involved in immune regulation. Amongst these is TRAIL which if absent results in autoimmune disorders in animals. TRAIL, however, is also shown to be capable of mediating neuronal and OGC injury [25,26]. Glatiramer acetate (GA) therapy results in generation of GA reactive T cells that are polarized toward the Th2 phenotype. We have shown that both Th1 and Th2 polarized T cells can transmigrate across a brain endothelial cell barrier [27]. Properties of such cells that could impact on tissue injury and repair would include their capacity to produce trophic factors and to promote microglia/macrophage mediated clearance of tissue debris [21,22]. The T cells implicated in the process of "protective autoimmunity" are mainly of the Th1 phenotype [28]. Intense immune-suppression regimens employed in experimental protocols that require subsequent immune stem cell rescue have been shown both to prevent subsequent disease relapses and to induce early loss of brain volume [29]. Recent experience showing that natalizamab, at least when used in combination with other imunomodulators, can result in activation of usually quiescent viruses (JC virus/progressive multi-focal leukoencephalopathy) indicates the importance of physiologic immune surveillance within the central nervous system.

12. Conclusion

The interaction of the constituents of the immune system with those of the nervous system is a dynamic process that can contribute to the injury or recovery processes that characterize MS. Therapeutic interventions targeting these interactions will need to continue to consider both the physiologic and pathologic aspects of such interactions.

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This process whereby non-specific effector responses could be directed to a specific target is referred to as antibody dependent cell cytotoxicity (ADCC).

6. αβ T cell mediated responses

Myelin reactive CD4 αβ T cells, particularly those with a Th1 phenotype, are the cell type usually used to adoptively transfer EAE and implicated as initiating the neuroinflammatory response in MS. Although such cells are shown to possess cytotoxic potential (reviewed in [9]), in vitro studies to date, indicate that human OGCs do not express MHC class II molecules and are not susceptible to MHC class II restricted lysis by myelin reactive CD4 T cells. A caveat is that such myelin reactive CD4 T cell when exposed to pro-inflammatory cytokines can be induced to up-regulate expression of molecules associated with non-specific cytotoxic cells (NK cells) and mediate non-MHC restricted target cell lysis. CD8 T cells, the classic cytotoxic cell population, are prominent in components of MS lesions. OGCs derived from the adult human CNS express MHC class I molecules that can be recognized by CD8 T cells. Our studies using CD8 T cells reactive to a specific peptide sequence of myelin basic protein (MBP) indicates that such cells can induce MHC class I restricted cytotoxicity of OGCs [10].

7. Target determined selective tissue injury

Active MS lesions contain a number of cell bound and soluble molecules that are capable of inducing tissue injury but lack the capacity to recognize cell lineage specific "markers". Selective target injury could still result dependent on whether such effectors acted by interaction with specific receptors that may be expressed only by selected cell types. Ligands for "death domain" containing receptors belonging to the tumor necrosis factor (TNF) receptor superfamily present in MS lesions include fas ligand, TRAIL and TNF itself. Such receptors are up-regulated in response to pro-inflammatory cytokines [11]. We have observed that human OGCs, especially when exposed to interferon (IFN)γ, express fas and undergo caspase dependent cell death within 4–6 h following exposure to fas ligand or activating anti-fas antibody. Fetal human CNS derived cortical neurons were resistant to fas signaled injury in vitro, a finding we attributed to reduced fas expression on these cells. Susceptibility to injury can also reflect intra-cellular properties including the signaling cascades induced by receptor engagement and/or the activity of cell survival genes. Fetal human CNS derived astrocytes express fas but are resistant to fas signaled injury [12]. Our studies suggest that such resistance can reflect either failure of fas engagement to initiate the caspase cascade or the presence of inhibitory molecules that block the cascade from completing the death program. Human OGCs become susceptible to TRAIL mediated cell death only if protein synthesis is blocked with cyclohexamide or if they receive an initial insult as we have modeled by introducing sublethal levels of p53 into these cells [13,14]. Progenitor OLGs appear to be more susceptible to TNF mediate compared to their mature counterparts [15]. Progenitor and adult OLGs also appear to differ with regard to expression of glutamate receptors [16].

8. Recovery from immune mediated injury

Functional recovery following an MS relapse likely reflects multiple factors. These include active or passive termination of the inflammatory response with reduction in soluble mediators. Functional MRI based studies indicate that cerebral re-organization also occurs. Histologic and MR based studies further document that remyelination does occur in the early inflammation dominated MS lesions. Most experimental data suggest that remyelination in the CNS is derived from progenitor cells that enter the lesion site and mature into myelin forming cells. Multi-potential and glial restricted progenitor cells have been detected in the adult human CNS, including in the region of MS lesions [17,18]. The injured CNS environment contains a multitude of molecules that can positively or negatively impact on the capacity of progenitor cells to survive, differentiate and migrate into lesion sites (reviewed in [19]). Immune mediators also can negatively or positively contribute to progenitor mediated remyelination. Progenitor cells may be selective targets of immune derived effector molecules including specific antibodies and glutamate [16,20]. Conversely, microglia/macrophages may play an integral role in removing tissue debris that inhibits the repair process [21]. The infiltrating immune response may further contain progenitor cells that can provide trophic support for neural progenitor cells [22]. Antibodies are described that can promote myelin formation or regeneration. Most tend to be of germ line origin rather than having undergone Ig gene rearrangement [23].

9. Recurrence of the disease process

A hallmark of the active MS lesion is the presence of microglia and macrophages that contain myelin debris, indicating that these cells can take up released myelin products. Such products could then be processed, transported in conjunction with MHC molecules to the cell surface, and be presented to lymphocytes present in the inflammatory milieu. This sequence of events could lead to an expansion of the array of myelin antigens to which the immune system is sensitized, a phenomenon referred to as determinant spreading. CNS released antigens can also be transported back to regional lymph nodes resulting in recurrent and expanded immune activation in the systemic compartment. Determinant spreading could also contribute to the wide diversity of the human myelin directed T cells found in MS patients,

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PERSPECTIVES IN PEDIATRIC PATHOLOGY

Periventricular Leukomalacia: Overview and Recent Findings

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ABSTRACT

Periventricular leukomalacia (PVL), the main substrate for cerebral palsy, is characterized by diffuse injury of deep cerebral white matter, accompanied in its most severe form by focal necrosis. The classic neuropathology of PVL has given rise to several hypotheses about the pathogenesis, largely relating to hypoxia-ischemia and reperfusion in the sick premature infant. These include free radical injury, cytokine toxicity (especially given the epidemiologic association of PVL with maternofetal infection), and excitotoxicity. Among the recent findings directly in human postmortem tissue is that immunocytochemical markers of lipid peroxidation (hydroxy-nonenal and malondialdehyde) and protein nitration (nitrotyrosine) are significantly increased in PVL. Premyelinating oligodendrocytes, which predominate in periventricular regions during the window of vulnerability to PVL (24 to 34 postconceptional weeks), are the targets of this free radical injury, and suffer cell death. Susceptibility can be attributed, at least in part, to a relative deficiency of superoxide dismutases in the preterm white matter, including premyelinating oligodendrocytes. Several cytokines, including interferon-γ (known to be directly toxic to immature oligodendroglia in vitro), as well as tumor necrosis factor-α and interleukins 2 and 6, have been demonstrated in PVL. Microglia, which express toll-like receptors to bacterial products such as lipopolysaccharide, are increased in PVL white matter and may contribute to the injury. Preliminary work suggests a role for glutamate receptors and glutamate transporters in PVL, as has been seen in experimental animals. These findings pave the way for eventual therapeutic or preventive strategies for PVL.

Key words: cerebral palsy, cytokines, excitotoxicity, oxidative stress, prematurity

INTRODUCTION

Periventricular leukomalacia (PVL) refers to damage of the immature cerebral white matter, occurring in the perinatal period. Pathologically, it is characterized by two components: (1) focal necrosis in the periventricular region and (2) diffuse reactive gliosis in the surrounding white matter. While they often occur together, the diffuse component is increasingly recognized to occur without the focally necrotic component. Each has a slightly different evolution: the necrotic foci, involving all tissue components (axons, glial cells), become cysts or focal glial scars; in contrast, the diffuse lesion results in a global delay in myelination, postulated to be due to preferential loss of premyelinating oligodendrocytes (OLs) in the immature white matter [1–3]. While the focal

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necrotic lesions correlate well with the motor deficits of cerebral palsy, the signs and symptoms attributed to the diffuse white matter lesion are broader and may include the cognitive and behavioral abnormalities observed in survivors of prematurity [4].

This review summarizes the clinical antecedents and neuropathologic features of human PVL and the results of recent experimental work beginning to shed light on mechanisms of injury at the cellular level.

CLINICAL FACTORS IN PVL

While PVL arises in full-term neonates, as well as infants as old as 2 postnatal months, the greatest period of risk is from 24 to 35 gestational weeks [5]. The clinical incidence of PVL is based largely upon epidemiologic studies using head ultrasound (HUS), with documentation of periventricular cysts. Because that method cannot detect the diffuse white matter changes alone, these studies likely underestimate the actual incidence of perinatal white matter damage. By HUS, reported incidences of "cystic" PVL in preterm infants born between 24 and 33 gestational weeks range between 5.7% and 16% [6–8]. Cystic PVL is also correlated with low birth weight, occurring in 2.3% of infants weighing 1750 grams, and in 3.2% of infants weighing 1500 grams [9]. In contrast, the autopsy incidence of PVL varies between approximately 20% and 75% [10, 11] (C. R. Pierson and colleagues, personal communication), depending on the age criteria for inclusion in the study (although in these reports, all infants were <38 weeks at birth).

Owing to recent advances in neonatal intensive care management of respiratory insufficiency, hemodynamic instability, and/or infection, the incidence of echolucencies (i.e., "cystic PVL") is decreasing [4]. Nevertheless, the incidence of cerebral palsy in prematurity remains unchanged and may reflect the importance of the "noncystic" component in the central white matter in producing long-term disability. Thus, the current concept of PVL must include not only the focal necrotic component in the periventricular region that typically evolves into cysts but also the diffuse, noncystic component in the central white matter [4], which is detectable as signal abnormality (e.g., diffuse excessive high signal intensity) by more sophisticated magnetic resonance imaging techniques [12,13]. As a consequence, many current investigations are now focusing on the pathology and mechanism of the diffuse white matter injury, as well as of the focal necrosis.

Aside from gestational age and weight at birth, risk factors for PVL are related to intrauterine infection, such as maternal urinary tract [7,14] or other infection [8], chorioamnionitis and/or premature rupture of membranes [5,6,9,15], and cardiopulmonary instability in the mother and/or fetus. Risks related to the latter include pregnancy, peripartum, and postpartum factors. First trimester hemorrhage is one pregnancy-associated factor [7]. Intrapartum factors comprise unstable fetal heart rate patterns, as documented by fetal monitoring [18,19]. Prolongation of pregnancy with tocolysis >24 hours [6] and with ritodrine [7] also increases the risk. In the neonate, acidosis at birth (pH < 7.2), meconium-stained amniotic fluid [7], low Apgar scores [17], and hyaline membrane disease [8] contribute to the risk for PVL. Postnatal treatment factors associated with PVL include longer periods of inotropic support [15] and of ventilation and oxygen inhalation [8,18]. Postnatal systemic hypotension occurs in up to 21% of preterm infants 1 750 grams at birth [9] and, because of poor cerebrovascular autoregulation in some babies, may result in cerebral hypoperfusion and white matter necrosis [19]. Stillborn fetuses also show PVL [20,21], along with chronic vascular changes, old infarction/abruption, and meconium staining in the placenta [20].

Anatomic and physiologic features of the immature central nervous system contribute to the risk of PVL. Based on injection studies in human postmortem material, relatively deficient vascularization of the periventricular white matter creates a "border zone" susceptible to hypoperfusion in the event of hypotension [22–24]. Doppler ultrasound data further indicate the occurrence, at least in some babies, of a "pressure-passive" circulation in the brain, such that systemic hypotension is not compensated by the usual cerebral vascular autoregulation seen in the mature brain [19]. The anatomic basis of this inability of the cerebral blood vessels to autoregulate is unclear.

NEUROPATHOLOGY OF PVL

Macroscopic pathology

The necrotic foci of PVL occur in the periventricular white matter within 1 to 2 cm of the

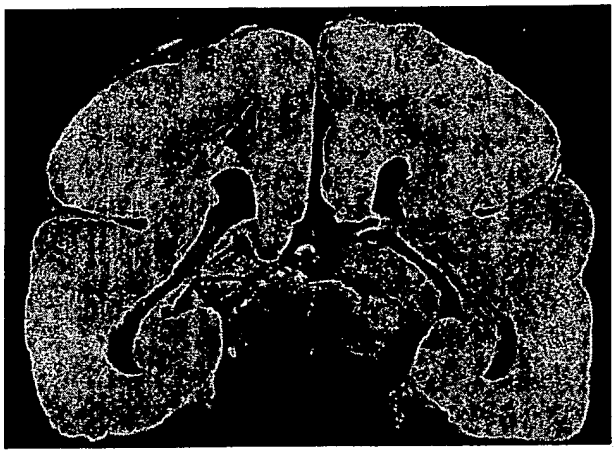

Figure 1. Macroscopic appearance of PVL. Note bilateral, cavitating lesions in the periventricular white matter of the parieto-occipital lobes in a 4-week-old infant born at 24 postconceptional weeks (reprinted from Kinney et al. [3] with permission); fortunately, such severe lesions are decreasing in incidence with improved neonatal intensive care.

ventricular wall, in all lobes, but most often at the level of the trigone and occipital horn, in the parieto-occipital lobe, including the optic radiations (Fig. 1). Typically, the necrotic foci measure 0.2 to 0.6 cm in diameter and, particularly if acute, may be overlooked on initial gross examination of the coronally sectioned hemispheres. Organizing foci may appear as "white spots" or chalky yellow lesions due to the presence of lipid-laden macrophages. Cavitation occurs within a few weeks, although some of the cysts may collapse to form a solid glial scar. In severe damage, periventricular cavitation persists and leads to an overall reduction in the cerebral white matter relative to the cerebral cortex, thinning of the corpus callosum, and ventriculomegaly. These latter features may also be seen following noncystic PVL, which may be visible acutely as dusky discoloration contrasting with pallor of the overlying cortical ribbon ("white ribbon sign" [25]).

Microscopic pathology

The evolution of the focal component of PVL begins initially with coagulative necrosis, characterized by complete tissue dissolution of all cell types, hypereosinophilia, nuclear pyknosis, and acutely necrotic, swollen axons (spheroids), visible on standard hematoxylin and eosin staining (Fig. 2A, Table 1). Within a week, the necrotic foci become organized with infiltrating macrophages (Fig. 2B, Table 1) and reactive astrocytes and rod-shaped cells (presumed to be microglia) at the margin [26]. Within a few weeks, resorption of the necrotic tissue results in periventricular cysts. In some cases, the cysts collapse and form focal glial scars, typically with residual entrapped lipid-laden macrophages and mineralized axons (Fig. 2C, Table 1). Diffuse white matter injury, which can be seen with and without focal necrosis, is characterized by prominence of reactive-appearing astrocytes, pyknotic or "acutely damaged" glia, and perivascular globules and/or mineralization (Fig. 2D). Immunohistochemically, the diffuse injury is characterized by microglial proliferation and gliosis, highlighted on CD68 and glial fibrillary acidic protein immunostains (Fig. 2E,F), preferentially involving the deep compared to intragyral white matter [2]. Axonal injury with spheroids is present at the margins of the necrotic lesions, and occasionally some distance away, and is confirmed by immunostaining for the human β-amyloid precursor protein, a marker of damaged axons [26,27].

Table 1. Maturation-dependent factors contributing to the vulnerability of human fetal white matter to glutamate, free radical, and cytokine toxicity

- Immaturity of the vasculature (long penetrating arteries) in the cerebral white matter of the human fetus, with the presence of deep (periventricular) vascular end-zones [11,22,24]
- Preferential sensitivity of developing oligodendrocytes (OLs) ($O4^+$, $O1^+$, MBP^+), as compared to mature (MBP^+) OLs, to free radical toxicity in rodent cell cultures [30]
- Developmental lag in the expression of the antioxidant enzymes Mn and CuZn superoxide dismutases compared to catalase and glutathione peroxidase in human fetal white matter [34]
- Preferential sensitivity of premyelinating OLs to the cytokine interferon-γ in rodent cell culture [59]
- Expression of IFN-γ receptor by human premyelinating OLs [44]
- Transient expression of AMPA[a] receptors in human premyelinating OLs [56]
- Transient expression of the GLT1 glutamate transporter in human premyelinating OLs [57]

Modified from Kinney et al. [3].
[a]AMPA indicates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

CELLULAR BASIS OF PVL

Animal models and in vitro studies, together with careful observations in human postmortem tissue, have increased our understanding of the cellular mechanisms underlying PVL.

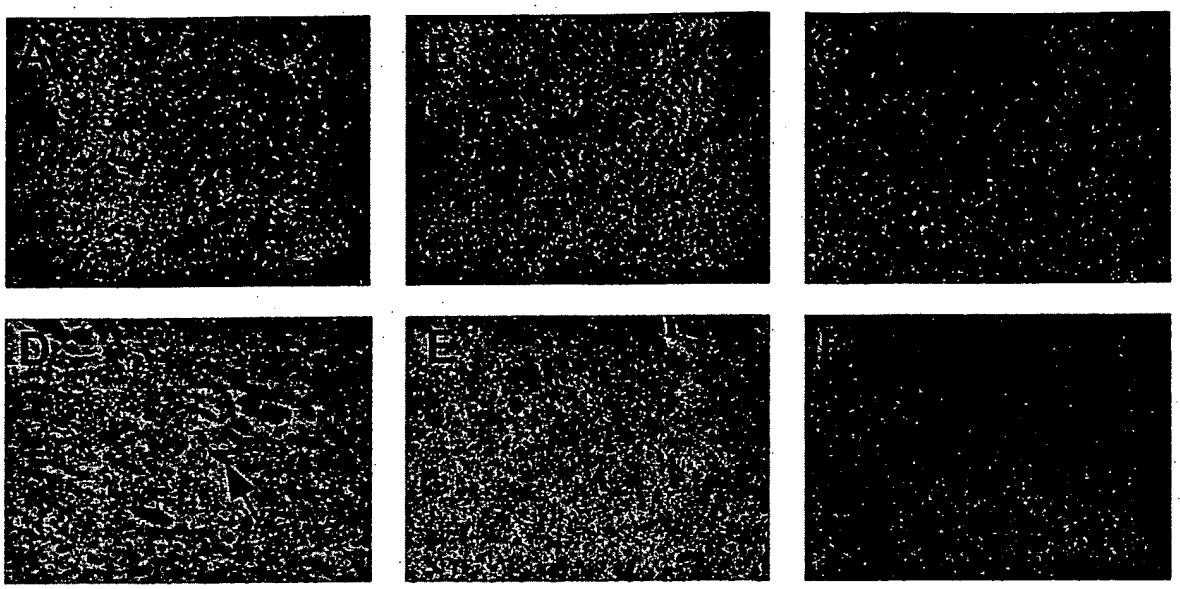

Figure 2. Microscopic appearance of periventricular leukomalacia (PVL). A. Acute white matter necrosis, identifiable as a geographic area of hypereosinophilia and nuclear pyknosis, in upper right (hematoxylin and eosin [H&E] stain, original magnification ×200). B. Organizing necrosis, with beginning cavitation and macrophage infiltrates (H&E stain, original magnification ×200). C. Healed or chronic PVL, with axonal loss, gliosis and mineralization (arrow) (H&E stain, original magnification ×200). D. Diffuse white matter injury, notable for prominent reactive astrocytes (arrow), pyknotic glial nuclei, and perivascular mineralization (H&E stain, original magnification ×400). E. Microglial proliferation in the diffusely injured white matter (CD68 immunostain, original magnification ×200). F. Reactive astrocytosis in the diffusely injured white matter (glial fibrillary acidic protein immunostain, original magnification ×200).

The recent application of special techniques directly in the human perinatal brain has contributed in a major way to our understanding of the cellular pathology of PVL. These techniques include single- and double-labeling immunocytochemistry and Western blotting to evaluate protein expression and in situ hybridization for messenger RNA expression. Given the significant association of PVL with cardiopulmonary compromise, as outlined above, the etiology of the white matter injury is considered to be hypoxic-ischemic, although maternofetal infection and inflammation are likely to play a role. The main pathogenetic mechanisms leading from hypoxia-ischemia to the development of PVL are (1) free radical injury, (2) inflammatory cells and mediators, and (3) excitotoxicity. While these factors are interrelated, they will be discussed separately.

Free radical injury

Our group recently utilized immunocytochemical markers to localize products of oxidative and nitrative injury, e.g., antibodies to 4-hydroxy-2-nonenal-protein adducts (HNE), malondialdehyde-protein adducts (MDA), and nitrotyrosine protein adducts (NT), to premyelinating OLs and astrocytes in the diffuse component of PVL (Fig. 3) [2]. 4-hydroxy-2-nonenal-protein adducts and MDA reflect lipid peroxidation of cell membranes and other lipid-containing cellular constituents by superoxide and hydrogen peroxide, whereas NT is formed from the action of reactive nitrogen species, chiefly the highly toxic peroxynitrite. Evidence of glial cell modification by these adducts supports the hypothesis that the cell injury in noncystic PVL is mediated by reactive oxygen and nitrogen species, likely resulting from ischemia followed by reperfusion. In addition, double-labeling immunocytochemical studies using premyelinating OL marker O4+ combined with terminal deoxytransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining for dying cells confirmed death of developing OLs in the diffuse component, as demonstrated in the brain of a premature infant at 35 weeks [2]. Interestingly, glial fibrillary acidic protein-immunoreactive astrocytes in the diffuse component are not labeled by the TUNEL method, suggesting that, despite evidence of lipid peroxidation and protein nitration, astrocytes are less vulnerable to these effects and subsequent cell death. Using CD68 for activated microglia/macrophages, we also found prominent activation of microglia in the diffuse

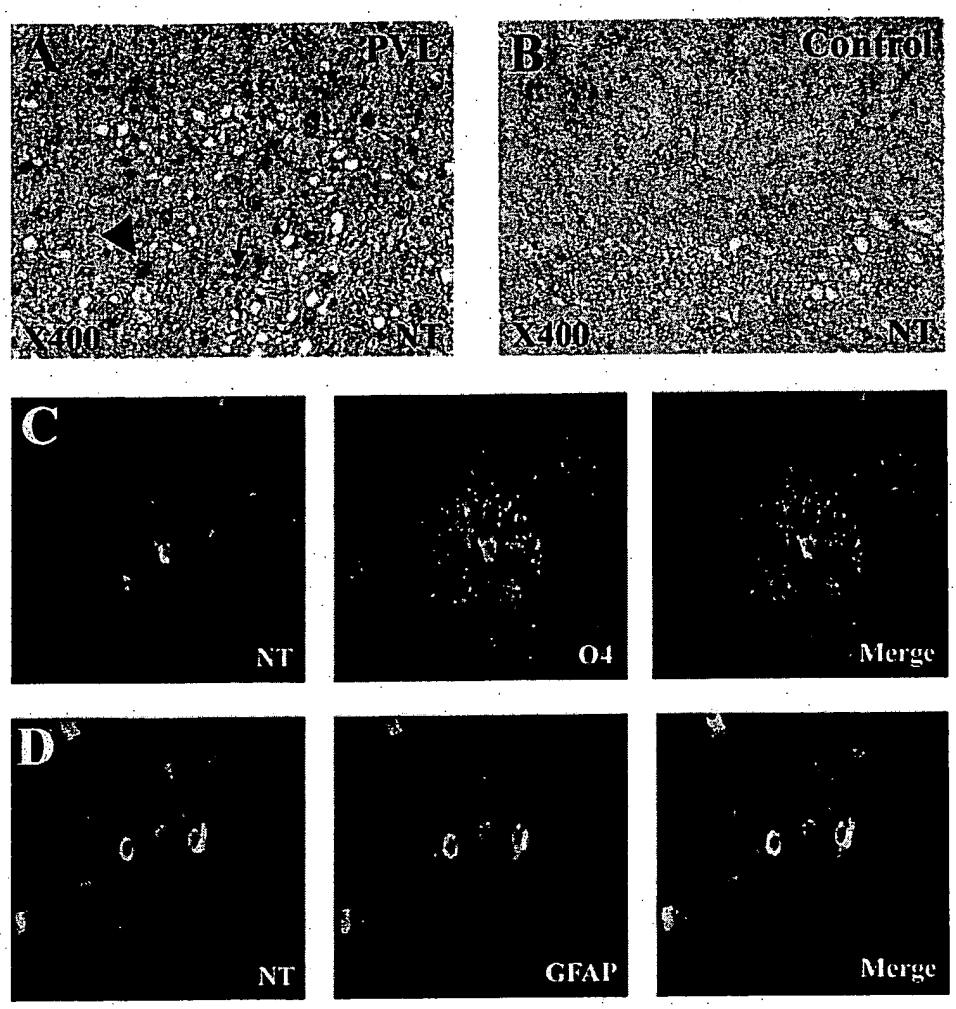

Figure 3. Markers of free radical injury in periventricular leukemia. A, B. Nitrotyrosine (NT) is present in diffusely gliotic white matter, in cells with morphologic characteristics of astrocytes (arrowheads) and oligodendroglia (arrows), but is absent in control tissue from a case of the same age (44 postconceptional weeks) (original magnification ×400). C, D. By immunofluorescent double-labeling, developing oligodendroglia (expressing O4) and reactive astrocytes (expressing glial fibrillary acidic protein [GFAP]) show evidence of nitration (original magnification ×600) (reprinted from Haynes et al. [2] with permission).

component of PVL, indicating an important and previously unrecognized association of these cells with white matter damage [2] (see Inflammatory Cells and Mediators, below).

Emerging from the human data is also a new appreciation for the topography of the free radical injury, in that the deep (periventricular) white matter shows greater involvement than does the superficial (intragyral and subcortical) white matter [2]. Likewise, the cortex is relatively, although not completely, spared [28]. This predilection is due not only to the vascular anatomic factors mentioned above but also to an intrinsic susceptibility of premyelinating OLs (the predominant cell type in the deep parieto-occipital white matter in the human during the PVL window [29]) to oxidative and nitrative injury.

From in vitro studies of rat OLs, it is established that premyelinating (O4+, myelin basic protein [MBP]–) cells are vulnerable, whereas mature (MBP+) cells are resistant to cystine deprivation (which leads to glutathione depletion and generation of oxygen free radicals) [30]. The basis for this difference, in part, is the expression of antioxidant enzymes in the latter. Recent work in our group has shown that mature (MBP+) OLs express higher levels of the antioxidant enzyme manganese-containing superoxide dismutase (MnSOD), which catalyzes the breakdown of superoxide to hydrogen peroxide and oxygen within mitochondria [31]. Moreover, introduction of MnSOD, via an adenoviral vector, into premyelinating OLs reduced cell death due to glutathione depletion [31]. MnSOD further appears to protect cells from injury due to reactive nitrogen species, including peroxynitrite [32]. Surprisingly, levels of copper-zinc–containing SOD, which catalyzes the dismutation of superoxide in the cytosol, did not differ between immature and mature OLs [31]. The other important antioxidant enzymes, glutathione peroxidase and catalase, which break down hydrogen peroxide (generated by the SODs) to water and

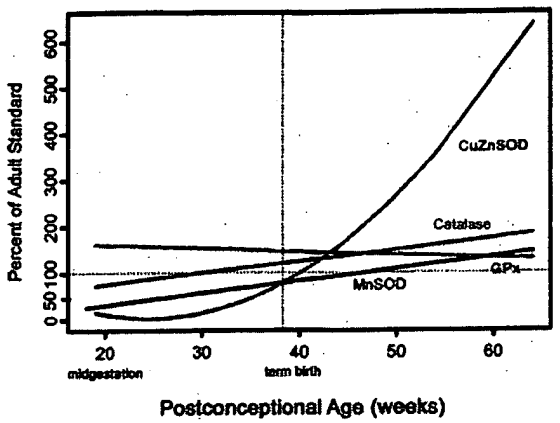

Figure 4. Expression of antioxidant enzymes in developing human white matter, relative to adult standard (horizontal dotted line at 100%). Note that levels of copper-zinc and manganese superoxide dismutases (CuZnSOD and MnSOD) lag behind those of catalase and glutathione peroxidase (GPx), which exceed adult levels, until after term birth (vertical dotted line) (reprinted from Folkerth et al. [35] with permission).

oxygen, show an interesting cooperativity in immature OLs [33]. While catalase expression is similar in developing and mature OLs, the former succumb to peroxide toxicity, whereas mature cells are resistant, owing to differential expression of glutathione peroxidase. The latter enzyme appears to protect catalase from inactivation by high levels of hydrogen peroxide [33].

While human stage-specific OL cultures are not available, double-labeling and Western blot protein expression profiling in the normal human white matter (i.e., without PVL) indicates a relative deficiency of antioxidant enzyme expression during the PVL window [34]. Specifically, by Western blot analysis of normal human white matter across development (from 18 to 214 postconceptional weeks), levels of catalase and glutathione peroxidase attain adult levels by 30 postconceptional weeks, while MnSOD and CuZnSOD levels do not reach adult expression until 40 weeks or later (Fig. 4) [34]. By single-labeling immunocytochemistry, glial cells expressed glutathione peroxidase and catalase as early as 21 postconceptional weeks, whereas SOD expression appeared after 27 weeks, again suggesting a disparity in the ability to break down sequentially superoxide to hydrogen peroxide, and then to water and oxygen. Furthermore, there appeared to be a qualitative increase in expression in the SODs, but not catalase, in mature as compared to developing OLs by double-labeling (Fig. 5), correlating with the in vitro data. Another finding from this study was that all antioxidant enzymes had higher-than-adult levels of expression during the peak period of postnatal myelin sheath synthesis in the parieto-occipital white matter (i.e., at 2 to 5 months of age), suggesting a need for maximal antioxidant capacity during high myelin lipid production.

Additional factors under investigation in the human include sources of nitric oxide (NO), such as nitric oxide synthase (NOS)-containing astrocytes, microglia, and subplate neurons [35]. These sources of NO could contribute to the observed NT seen in astrocytes and OLs in human PVL [2].

Inflammatory cells and mediators

Microglia are increasingly recognized to have a central role in the pathogenesis of many neurologic conditions, including PVL. We reported a significant increase in the density of CD68-immunoreactive microglia in the diffuse component of PVL (Fig. 2E) and that this increase was correlated with increased staining for markers of oxidative and nitrative stress [2]. Activated microglia and macrophages are significant sources, as well as targets, of cytokines, which orchestrate cellular immune and inflammatory responses in the brain and are known to be upregulated in response to both hypoxia-ischemia and infection. In PVL, ascending intrauterine infection and choroiamnionitis are thought to initiate a maternal cytokine response, leading to transplacental passage of cytokines to the fetus. It is also possible for the fetus to generate its own cytokine response ("fetal inflammatory response"). In either case, entry of cytokines into the fetal brain may occur via the blood-brain barrier, resulting in cytokine toxicity to vulnerable regions of the brain, particularly the white matter [36]. Induction of cytokine production within the brain can also occur as a result of exposure of the brain to circulating bacterial antigens [37]. These may enter the brain and stimulate local production of cytokines by brain microglia; these cells in turn may be stimulated to produce other molecules, e.g., NO, that are directly toxic to OL precursors. Activated microglia have recently been found to express toll-like receptors (TLRs), which recognize molecular motifs common to many different microorganisms, as part of the innate immune response [38]. In particular, TLR4, which recognizes bacterial lipopolysaccharide, has been shown to be present on microglia in the human (adult) brain [39] and is necessary for

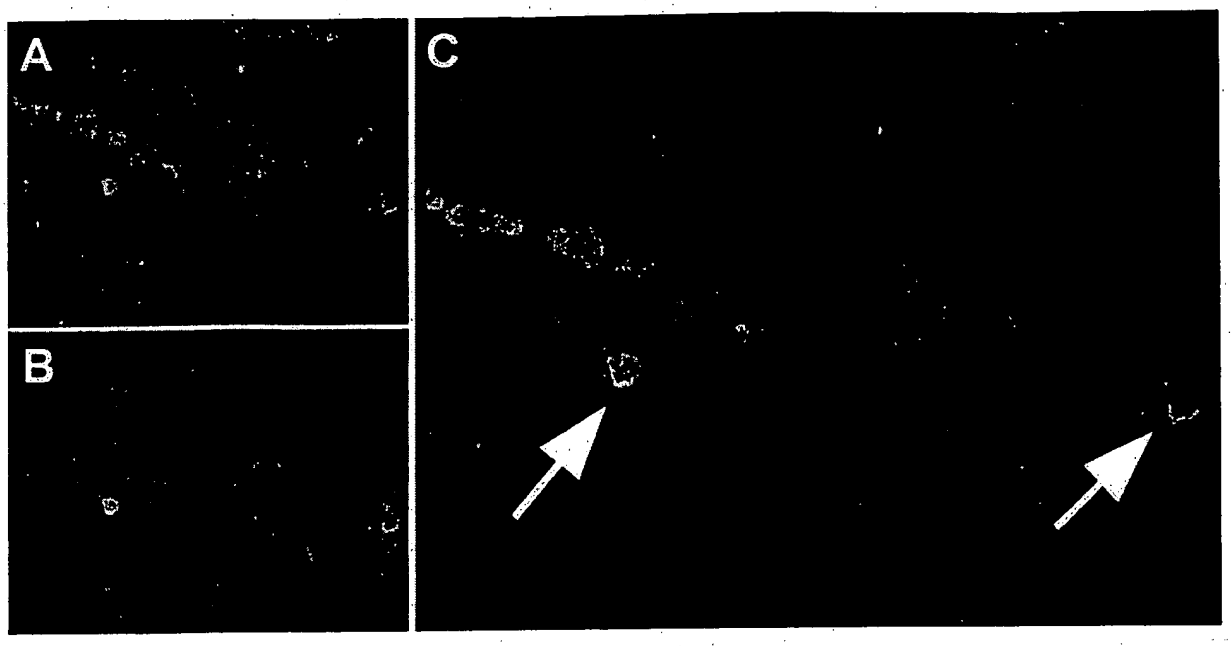

Figure 5. Colocalization of manganese-containing superoxide dismutase (MnSOD) (A, green) in developing oligodendroglia expressing the O4 surface marker (B), visualized by immunofluorescent double-labeling (C, merged image, arrows) (original magnification ×400), beginning after about 29 postconceptional weeks (streak in A and C is MnSOD blood vessel just out of plane of focus).

lipopolysaccharide-induced injury to OLs [40] as well as neurons [41]. However, the role of TLR4 in human PVL remains to be defined.

The potential importance of microglia in PVL is highlighted by recent work demonstrating a significant, transient, development-associated increase in the density of activated microglia in periventricular white matter from 20 to 54 postconceptional weeks in the human [42]. This exciting finding suggests not only a possible role

Table 2. Summary of the simultaneous evolution of the histopathology of the focal and diffuse components of periventricular leukomalacia

Stage	Timing	Focal component	Diffuse component
I. Acute (noninflammatory)	8 to 24 hours	Coagulative necrosis; β-amyloid precursor protein-positive axonal spheroids; dissolution of all cellular elements	Injury to $O4^+$ and $O1^+$ OLs (TUNEL[a]-positivity); ? diffuse axonal injury from free radicals, cytokines, glutamate
II. Subacute/organizing (inflammatory)	2 to 7 days	Macrophage infiltration; rim of reactive astrocytes; ↑ cytokines in macrophages; axonal spheroids; beginning cyst formation	Reactive astrocytes and activated microglia; ↑ cytokines; ROS and RNS markers in $O4^+$ and $O1^+$ OLs and reactive astrocytes; dying (TUNEL-positive) $O4^+$ and $O1^+$ OLs; loss of O4 and O1 cells; ?injury to subplate neurons
III. Chronic	Weeks to months	Continuation of stage II; evolution of cyst, which can persist or collapse to form focal glial scar; axonal mineralization	Reactive astrocytes and microglia; delayed myelination; venticulomegaly, corpus callosum thinning; subtle secondary abnormalities in the development of overlying cortical neurons
IV. Recovery/repair	Weeks to months	End-stage periventricular cysts and/or focal glial scars	? Repopulation of $O4^+$ and $O1^+$ OLs; appropriate or, if severe, permanently deficient myelination

Modified from Kinney et al. [3].
[a] See text for definition.

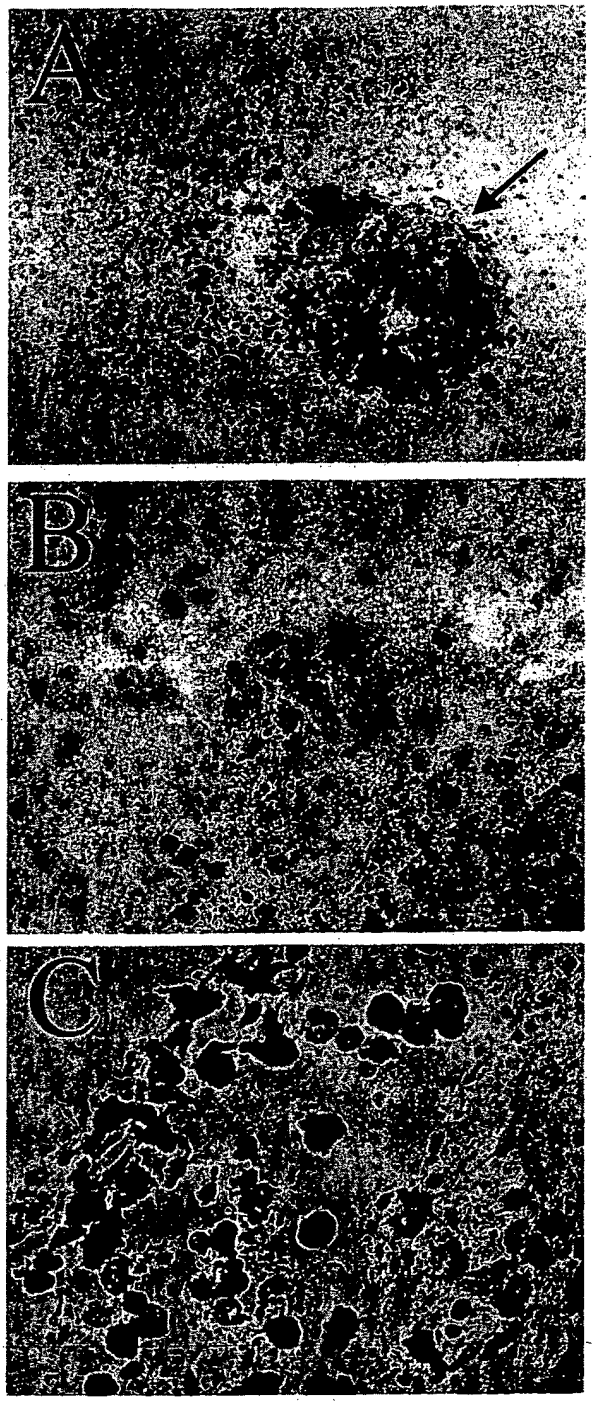

Figure 6. Cytokine expression in periventricular leukomalacia. A, B. Interferon (IFN)-γ is present in necrotic foci (arrow), in cells with the morphologic characteristics of macrophages. C. CD68 immunostain on an adjacent section confirms the identity of the IFN-γ–positive cells as macrophages (reprinted with permission from Folkerth et al. [45] with permission).

for microglia in normal development, such as axon outgrowth (also known to be actively occurring during this developmental interval [43]), but also a potential "priming" effect on white matter injury, should exposure to hypoxia-ischemia and/or infection occur during this time.

Several cytokines, including interferon-γ (IFN-γ) [44], tumor necrosis factor-α (TNF-α) [45,46], and interleukin-2 [48] and -6 [47] have been detected directly in human PVL tissue. For IFN-γ, expression was found in cells with morphologic features of oligodendroglia, astrocytes, and, in the necrotic foci of PVL, macrophages (Fig. 6). Surprisingly, rod-shaped microglia did not express IFN-γ. Using a semiquantitative grading scheme, an increasing grade of expression of IFN-γ was noted to correlate with the density grade of GFAP-positive astrocytes, but not CD68-positive microglia [2,44]. It also correlated with the grade of malondialdehyde adduct expression, a marker of oxidative injury, in PVL [2]. Given that IFN-γ is directly toxic to immature OLs [48] and that it induces NOS activity in astrocytes [49], as well as proliferation of astrocytes and microglia [49,50], this cytokine is likely to play a critical role in the white matter injury of PVL, particularly the diffuse component. Of note, the IFN-γ receptor was expressed on developing OLs, indicating particular vulnerability to this cytokine [44]. The potentiation by TNF-α of IFN-γ–mediated injury to developing oligodendroglia [51] suggests an important role as well for TNF-α in PVL, and, in fact, has been demonstrated in macrophages and reactive astrocytes [45,46].

Excitotoxicity

While all cells of the body are subject to the effects of free radical and cytokine toxicity, the central nervous system is unique in that excitotoxic injury can result from exposure to excessive levels of the excitatory amino acid neurotransmitter glutamate. Excitotoxicity is a key factor in neuronal injury mediated by N-methyl-D-aspartate (NMDA) and non-NMDA (kainate [KA] and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA]) receptors. More recently, however, non-NMDA (but not NMDA) receptors have been identified on premyelinating and mature OLs, strongly implicating glutamate toxicity as a contributor to OL injury and death following hypoxia-ischemia (reviewed in [4,52]).

In PVL, several factors likely are at play in the accumulation of excessive extracellular glutamate. Leakage of glutamate from damaged axons (visible as axonal spheroids in the periventricular

foci of necrosis) is one source of increased extracellular glutamate in hypoxia-ischemia. Additionally, regulation of the extracellular glutamate concentration by glutamate transporters, which are high-affinity, sodium-dependent pumps found on oligodendroglia, astrocytes, axons, and possibly microglia [53], is disturbed, leading to paradoxical reversal of GLT1 (glutamate transporter) function. Glutamate is then transported out of these cells, markedly increasing the extracellular concentration. Cytokines can also cause inhibition of glutamate transport [54]. The excess glutamate leads to prolonged depolarization of the AMPA and KA receptors on the cell surface membrane of premyelinating OLs, leading to influx of calcium and sodium, respectively, contributing to calcium-induced cell injury, as well as sodium-induced cell swelling, and the generation of oxygen free radicals through the promotion of cystine efflux and depletion of glutathione [55]. In premyelinating OLs, it is also postulated that excess calcium upregulates NOS, the synthetic enzyme for NO and, in turn, increased production of NO and peroxynitrite, the latter an especially potent biological oxidant.

Our group has recently demonstrated AMPA receptors and the GLT1 glutamate transporter on premyelinating OLs by immunocytochemistry in the periventricular white matter of the developing human [56]. In particular, the AMPA receptors GluR4 are increased and GluR2 are decreased, during the window of vulnerability to PVL, in comparison with term expression levels. Since GluR2-lacking receptors allow calcium influx, the makeup of the receptors during this age interval increases the susceptibility to excitotoxicity in the event of high levels of extracellular glutamate, such as following hypoxia-ischemia [56]. Preliminary results also suggest that the glutamate transporter GLT1 is overexpressed in the premyelinating OL during the window of vulnerability to PVL, also thereby contributing to excitotoxicity as a mechanism for white matter injury [57].

Miscellaneous

Glial cells around necrotic foci in PVL have been found to express the myelin transcription factor 1 (MyT1), while the same regions contained increasing MBP and proteolipid protein immunoreactivity [58]. MyT1 is a zinc-dependent, DNA-binding protein that is expressed in early OL progenitors, and its presence in PVL lesions may be indicative of attempted myelin repair [58].

CONCLUSION AND FUTURE DIRECTIONS

Based upon the findings of classic neuroanatomic and neuropathologic studies, as well as clues from animal models and stage-specific OL cultures, recent work directly in human postmortem tissue has begun to elucidate the pathogenetic mechanisms likely to be involved in the susceptibility to and development of perinatal white matter injury. These are the first steps to designing and implementing therapeutic and preventive strategies for PVL, to be used in the neonatal intensive care nursery.

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CME

White matter hyperintensities and neuropsychological outcome following carbon monoxide poisoning

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Abstract—*Background:* Carbon monoxide (CO) poisoning may result in white matter hyperintensities (WMH) and neurocognitive impairments. *Objective:* To assess in a prospective study WMH in CO-poisoned patients and their relationship to cognitive functioning. *Methods:* Seventy-three consecutive CO-poisoned patients were studied. MR scans and neurocognitive tests were administered on day 1 (within 36 hours after CO poisoning), 2 weeks, and 6 months. Age- and sex-matched control subjects for white matter analyses only were obtained from the authors' normative imaging database. MR scans were rated for WMH in the periventricular and centrum semiovale regions, using a 4-point rating scale. Two independent raters rated the scans, and a consensus was reached. *Results:* Thirty percent of CO-poisoned patients had cognitive sequelae. Twelve percent of the CO-poisoned patients had WMH, with significantly more periventricular, but not centrum semiovale, WMH than control subjects. The WMH in CO-poisoned patients did not change from day 1 to 6 months. Centrum semiovale hyperintensities were related to worse cognitive performance. Duration of loss of consciousness correlated with cognitive impairment at all three times. Initial carboxyhemoglobin levels correlated with loss of consciousness but not with WMH or cognitive sequelae. *Conclusions:* CO poisoning can result in brain injury manifested by WMH and cognitive sequelae. The WMH were not related to CO-poisoning severity. The WMH occurred in both the periventricular and the centrum semiovale regions. However, only those in the centrum semiovale were significantly associated with cognitive impairments.

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White matter hyperintensities (WMH) refer to unidentified bright objects observed on T2-weighted or mixed weighted brain MR images as increased signal intensity. WMH often correspond to changes described as leukoaraiosis on CT.[1,2] Possible etiologies of WMH include dilated perivascular spaces, arteriolosclerosis, partial loss of myelin and axons, gliosis, and lacunar infarction.[2] Two types of WMH are often distinguished: periventricular WMH (PVWMH) that border the lateral ventricles[3] and centrum semiovale WMH (CSWMH) or deep WMH located in the subcortical white matter. There is some evidence suggesting that there are differences in both the physiologic basis and the clinical significance of the PVWMH and CSWMH.[4-7]

WMH have been associated with decreased mental processing speed, decline in executive functioning,[2] and balance and gait abnormalities.[8] Increased prevalence of WMH has been reported in paranoid psychosis,[9] bipolar disorder,[10] depression,[11] AD and vascular dementia,[12] and carbon monoxide (CO) poisoning.[13]

CO poisoning is the most common cause of poisoning morbidity and mortality in the United States.[14] Acute CO poisoning can cause loss of consciousness, cognitive impairments, neurologic sequelae, coma, and death.[14] Cognitive sequelae following CO poisoning include impaired memory, attention, visual spatial skills, mental processing speed, executive function, apraxia, parkinsonian symptoms, and dementia.[15-17] Mechanisms of brain injury following CO exposure include hypoxia,[18,19] reduced cellular O_2 metabolism,[20,21] lipid peroxidation leading to oxidative injury,[22] damage to the vascular endothelium due to deposition of peroxynitrate,[23] excitotoxicity,[24] and apoptosis.[25,26]

CO poisoning can cause acute demyelination, damage to subcortical white matter, globus pallidus, thalamus, and hippocampus,[27] as well as generalized cortical atrophy.[15] Brain CT and MR following CO poisoning may show lesions of the globus pallidus, putamen,[28] caudate nucleus,[28] thalamus,[29] and substantia nigra[30] and cortical, corpus callosum,[31] for-

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nix,[32] and hippocampal atrophy.[15] CO poisoning results in white matter changes including lesions in the cerebellar white matter[33] and subcortical and periventricular white matter.[34] White matter and globus pallidus lesions have been reported to predict neurologic outcome following CO poisoning.[35,36]

WMH occur following CO poisoning, but inferences that can be drawn from these studies are limited owing to 1) patient selection bias, 2) small sample size, 3) lack of control subjects, and 4) variable time from CO exposure to imaging.[36-38] In an effort to clarify the relationship between CO poisoning and WMH, we assessed consecutive CO-poisoned patients and age- and sex-matched normal control subjects for WMH in the periventricular and centrum semiovale regions on brain MR using a prospective within-subjects and between-subjects design. We also examined the CO-poisoned patients' cognitive function using a brief neuropsychological test battery. We hypothesized that the CO patients would have more WMH in both the periventricular and the centrum semiovale regions than control subjects and that WMH would be associated with cognitive sequelae.

Methods. *Subjects.* The Hyperbaric Medicine Service at LDS Hospital recruited CO-poisoned patients from February 1994 through July 1996 for this study. Patients were referred from emergency departments in Utah, Idaho, and Wyoming. Patients were excluded if they were ≤16 years old, consent was not possible (e.g., patient comatose and no family available), they were pregnant, or death was judged to be inevitable. There were 135 eligible CO-poisoned patients, of whom 73 patients were enrolled; 62 patients declined the study. Reasons for study refusal included not interested in study (n = 21), cost of transport to our hospital or treatment costs (n = 17), inconvenience (n = 14), not able to complete follow-up (i.e., out of state) (n = 5), and referring physician did not refer patient to the study (n = 5). Patients were eligible if they 1) had a diagnosis of CO poisoning on the basis of history and 2) had an elevated carboxyhemoglobin (COHb) level of ≥10%. Patients with a COHb level of <10% were included if CO poisoning was the only possible diagnosis. Seventy-three CO-poisoned patients were prospectively enrolled in our study. This study had institutional review board approval, and informed consent was obtained from the patient or a legal surrogate. Included CO-poisoned patients had a mean COHb level of 22.0 ± 10.6% (range 1.2 to 39.0%), CO exposure duration (n = 57) of 15.2 ± 47.5 hours (range 0.25 to 308 hours), and base excess (n = 47) of −1.6 ± 3.6 mEq/L (range −14.3 to 2.6 mEq/L). All patients were treated with high-flow oxygen by nonrebreathing reservoir facemask.

The CO-poisoned group comprised 49 males and 24 females with a mean age of 34.7 ± 13.6 years (range 16 to 86 years) with a mean educational level of 12.1 ± 2.8 years (range 2 to 20 years). Control subjects for MR white matter analysis only were selected from a normative database[39] and were age and sex matched to the CO patients. Each control subject was within 2 years of the age of his or her matched CO-poisoned patient (mean age 35.0 ± 13.4 years). The control subjects were a priori excluded if they had a prior history of head injury with loss of consciousness, neurologic disease, psychiatric disorder, or history of alcohol or drug abuse.[39] Loss of consciousness occurred in 52% of the CO patients at the time of CO exposure, and five patients were intubated. The duration of unconsciousness for each patient was coded on a scale of 0 to 9 (see table 1). Mean CO exposure duration was 22 ± 70 hours (range 15 minutes to 20 days). Causes of CO exposure included exposure to automobile exhaust (42%) (half [21%] were intentionally poisoned), internal combustion engine exhaust (29%), faulty furnaces (22%), and fumes from smoldering charcoal briquettes (6%) and from fire (1%). A board-certified neurologist independently reviewed all scans for the presence of abnormalities such as infarcts or lesions in the basal ganglia. There were no gross abnormalities identified. One patient had bilateral lesions of the basal ganglia at 2 weeks, which had resolved at 6 months.

Table 1 Duration of loss of consciousness (LOC) in the carbon monoxide-poisoned subjects

LOC code	Duration of unconsciousness	No. of subjects	%
0	None	34	46.6
1	<1 min	4	5.5
2	1–5 min	9	12.3
3	5–10 min	4	5.5
4	10–30 min	7	9.6
5	30–60 min	4	5.5
6	1–6 h	5	6.8
7	6–12 h	1	1.4
8	12–24 h	4	5.5
9	>24 h	1	1.4

Neuropsychological tests. The CO-poisoned patients were administered a neuropsychological test battery consisting of digit span, digit symbol, and block design from the Wechsler Adult Intelligence Scale–Revised,[40] Trail Making Test Parts A and B,[41] and story recall from the Denman Neuropsychology Memory Scale.[42] The neuropsychological tests were administered on the day of CO poisoning (day 1) and at 2 weeks and 6 months after CO poisoning.[32]

T-scores corrected for age, sex, and education were used for data analysis (mean = 50, SD = 10).[43,44] Cognitive impairment is defined as the number of SD less than the *T*-score of 40 for Trail B, block design, and digit symbol. An overall cognitive impairment score was calculated by summing the number of SD for each test that were >1 SD below the mean. For the purpose of statistical analysis comparing WMH with cognitive function, only Trail B, block design, and digit symbol *T*-scores were used.

Unfavorable cognitive sequelae were defined as present if any neuropsychological subtest score was >2 SD below the mean (or if at least two subtest scores were each >1 SD below the mean) using demographically corrected *T*-scores. If the patient reported memory, attention, or concentration difficulties, cognitive sequelae were considered present if a neuropsychological subtest score was >1 SD below the mean or if two subtest scores each were >0.5 SD below the mean.

Five patients were unconscious and intubated on day 1 and were unable to be tested; therefore, their neuropsychological data were imputed. The day 1 scores were imputed using the mean of the day 1 scores of patients with cognitive sequelae. Likewise, one patient remained intubated at 2 weeks, so scores were imputed using the mean of the 2-week scores of patients with cognitive sequelae.

Imaging and analysis. The MR scans were obtained within the first 24 to 36 hours (day 1) following CO poisoning, at 2 weeks, and at 6 months following the CO poisoning. Both the CO patients and the control subjects were imaged supine with the head in a fixed position, using the same scanner and scanning protocol. Sagittal and spin echo axial images were collected on a 1.5 T quadrature head coil GE Signa Scanner (General Electric, Milwaukee, WI). Sagittal scans were T1-weighted with 500/11/2 repetition time (ms)/echo time (ms)/excitations, a 256 × 192 pixel acquisition matrix, and a field of view (FOV) of 22 cm. Sagittal images were 5 mm thick with a 1-mm interspace gap. Axial intermediate and T_2-weighted (3,000/31; 90/1 repetition time [ms]/echo time [ms]/excitations) spin echo images were acquired with slice thickness of 5 mm and 2-mm interspace gap, FOV of 22 cm, and acquisition matrix of 256 × 192. The scan range extended from the most inferior point of the cerebellum to the most superior point of the cerebral cortex on the midsagittal image. Imaging data remained in digital form throughout the analysis.

The WMH were rated based on a 4-point semiquantitative rating method.[45] The original method used a 8-point scale. The current study used a modified rating method with a 4-point scale, with values of 0, 0.5, 1, and 2. PVWMH and CSWMH were rated by comparing the patient's MRI scan with sets of PVWMH and CSWMH standard images. A rating of 0 was assigned to images with no hyperintensities, 0.5 for WMH of less than or equal to Standard 1, 1 for WMH definitely more than Standard 1 and less than or equal to Standard 2, and 2 for WMH definitely greater than Standard 2.[45] The PVWMH were assessed on the first MR scan that was rostral to the lateral bulges of the heads of the caudate nuclei. The CSWMH were assessed on the MR scan that showed the lateral ventricles at the point of the longest anteroposterior connection.[45]

The PVWMH and CSWMH were rated on the patients' T2-weighted and proton density images.[45] All scans were rated for PVWMH and CSWMH, first with right and left side ratings, then with a combined rating that assessed WMH bilaterally. Because no significant differences were found between right and left WMH ratings, only the combined ratings were used in the statistical analysis. Inter-rater reliability was first established with a series of 29 scans and then applied to all scans in the study. In all cases, inter-rater reliability (Pearson product–moment correlations) exceeded 0.9.

The CO patients' three scans and control subjects' scans were randomly mixed and were labeled with a random identification number. The scans were analyzed with the raters' blind to the patient identity, scan date, and group (CO patients and normal control subjects). Two raters rated the scans independently, the raters discussed discrepancies, and a consensus was reached by referring back to the original scans (while still blinded). The ratings were

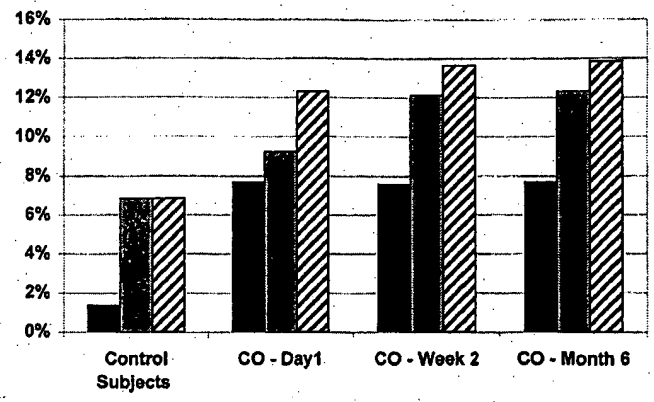

Figure 1. The percent with white matter hyperintensities in control subjects and carbon monoxide (CO)-poisoned patients at day 1 (n = 65), 2 weeks (n = 66), and 6 months (n = 65). Black columns = patients with periventricular white matter hyperintensities; gray columns = patients with centrum semiovale white matter hyperintensities; striped columns = patients with any white matter hyperintensities.

examined to determine if the WMH differed for each patient over time. For the CO-poisoned subjects with WMH, all three MR scans were viewed unblinded to determine if the WMH were stable over time. If differences were found across the three time points, the CO patients' scans were re-examined by the raters to determine if the WMH had changed over time or if differences in ratings were due to variation in scan quality, slice location, or angle of imaging.

Statistical analysis. Paired samples *t*-tests were used to compare CO-poisoned subjects' day 1 PVWMH and CSH ratings with control subjects' PVWMH and CSWMH ratings. *T*-tests were used to compared CO-poisoned patients who lost consciousness with those who did not lose consciousness for cognitive impairments, Trails B, and block design. To assess the effect of white matter changes over time (day 1, 2 weeks, and 6 months) on PVWMH and CSWMH ratings, repeated measures analysis of variance was carried out. Pearson's correlations were used to correlate PVWMH and CSWMH ratings with age, initial COHb levels, duration of loss of consciousness, and duration of CO poisoning. Point biserial correlations were used to correlate loss of consciousness (either yes or no) with PVWMH and CSWMH ratings. Pearson's correlations were used to correlate PVWMH and CSWMH ratings with neuropsychological test scores (Trails B, block design, and digit symbol) at day 1, 2 weeks, and 6 months. Half-normal plots were carried out to check for spurious correlations, and the results showed that the correlations were different from zero.[46]

Results. The CO-poisoned patients (n = 9; 12%) had more WMH (PVWMH and CSWMH) than control subjects (n = 5; 7%). There were higher PVWMH ratings for the CO subjects on day 1 than for the control subjects (figure 1) ($t[1,64] = 2.05$, $p = 0.04$). The CO-poisoned patients had more PVWMH on day 2 than the control subjects ($t[1,64] = 2.05$, $p = 0.04$). Five CO-poisoned patients and one control subject had PVWMH on day 1, all with 0.5 ratings. There were no differences between CSWMH for the CO-poisoned

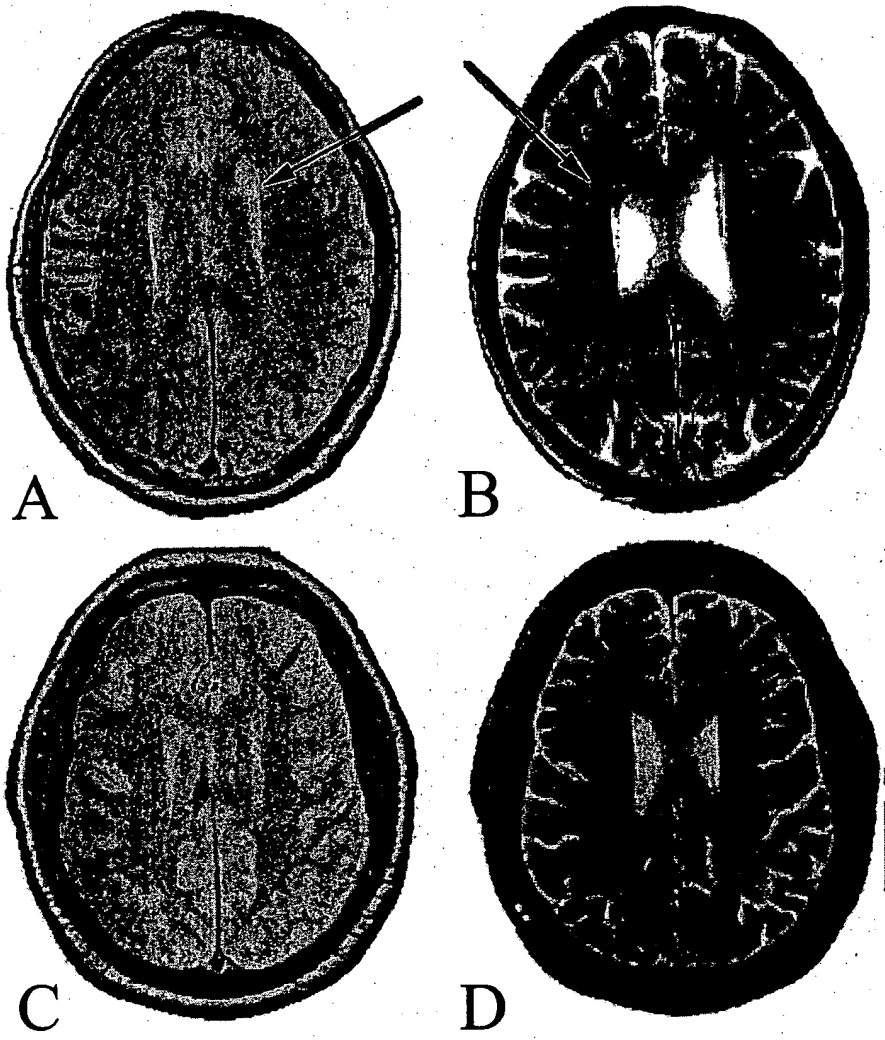

Figure 2. Axial images of carbon monoxide (CO)-poisoned patients (A,B) and matched control subjects (C,D): proton density (A,C) and T2-weighted (B,D) images. The arrow in A shows a representative periventricular white matter hyperintensity; the arrow in B shows a representative centrum semiovale white matter hyperintensity in a CO-poisoned subject.

patients on day 1 compared with control subjects (see figure 1). Six CO-poisoned patients and five control subjects had CSWMH on day 1 with ratings of 0.5, except one 86-year-old CO patient with a rating of 1.0. Representative MR scans of CO patients with PVWMH and CSWMH can be seen in figure 2. No significant differences were found for PVWMH or CSWMH between day 1 and 2 weeks, between day 1 and 6 months, or between 2 weeks and 6 months.

WMH related to demographic and CO poisoning variables. Patients with self-inflicted CO poisoning did not differ from those with accidental CO poisoning for PVWMH and CSWMH. Age correlated with both PVWMH (correlation coefficient $r = 0.43$, $p < 0.0005$) and CSWMH (correlation coefficient $r = 0.60$, $p < 0.0005$). Because there was one 86-year-old CO-poisoned patient whose inclusion might have confounded the age effect owing to WMH and cognitive changes normally associated with aging, the age analysis was repeated with this subject removed from the analysis. Age (minus the 86-year-old patient) remained correlated with the PVWMH and CSWMH ($r = 0.29$, $p < 0.02$ to $r = 0.50$, $p < 0.0001$). There were no significant correlations between loss of consciousness or duration of unconsciousness and WMH. There were no significant correlations between WMH and COHb level and duration of CO exposure at any of the three scan times.

WMH related to neuropsychological impairments. Thirty percent of the CO patients had cognitive sequelae. The PVWMH were not related to any cognitive measure. The CSWMH correlated with Trails B at 2 weeks ($r = -0.26$, $p = 0.03$) and 6 months ($r = -0.27$, $p = 0.03$). The CSWMH correlated with cognitive sequelae on day 1 ($r = 0.30$, $p = 0.02$), 2 weeks ($r = 0.28$, $p = 0.02$), and 6 months ($r = 0.26$, $p = 0.04$). Block design and digit symbol were not related to CSWMH.

Neuropsychological impairments related to demographic and CO poisoning variables. Unconsciousness and being intubated (n = 5) on day 1 correlated with block design ($r = 0.41$, $p < 0.0005$) and overall cognitive impairment scores ($r = 0.44$, $p < 0.0005$) at 6 months. Duration of unconsciousness of 5 minutes or longer was associated with worse cognitive outcome by risk factor analysis.[47] Forty-seven patients (64%) were unconscious for <5 minutes and 26 patients (36%) were unconscious for periods ranging from >5 minutes to 24 hours (see table 1). Patients with unconsciousness for >5 minutes compared with <5 minutes had worse overall cognitive impairment scores at day 1 ($t[1,69] = 3.7$, $p > 0.001$), 2 weeks ($t[1,70] = 2.5$, $p = 0.01$), and 6 months ($t[1,68] = 2.4$, $p = 0.02$). Patients with unconsciousness for >5 minutes compared with <5 minutes were worse on Trails B at day 1 ($t[1,69] = 2.6$, $p =$

0.01) and 6 months ($t[1,68] = 1.9$, $p = 0.05$) and on block design at 6 months ($t[1,69] = 2.4$, $p = 0.02$).

Loss of consciousness was correlated with the initial COHb level ($r = 0.28$, $p = 0.02$). The initial COHb level was inversely correlated with the duration of CO poisoning ($r = -0.30$, $p = 0.01$). Males had a higher mean initial COHb level (n = 49; mean = 24.7 ± 8.6%) than females (n = 24; mean = 15.4 ± 12.5%; $t[1,71] = -3.8$, $p < 0.0005$). Females had a longer mean duration of poisoning (mean = 52.8 ± 115.5 hours) than males (mean = 6.5 ± 14.5 hours; $t[1,71] = 2.78$, $p = 0.007$). The duration of unconsciousness correlated with day 1 overall cognitive impairment scores ($r = 0.40$, $p = 0.001$), block design ($r = -0.25$, $p = 0.04$), and Trails B ($r = -0.25$, $p = 0.04$). Duration of unconsciousness also correlated with overall cognitive impairment ratings at 2 weeks ($r = 0.28$, $p = 0.02$) but not at 6 months. Age correlated with Trails B at 2 weeks ($r = -0.28$, $p = 0.02$) and 6 months ($r = -0.26$, $p = 0.03$).

Discussion. This prospective MR study assessed WMH in a large group of consecutive CO-poisoned patients compared with age- and sex-matched control subjects. In our group of CO-poisoned patients, 30% developed cognitive sequelae and 12% had WMH. The cognitive impairments included impaired executive function and slow mental processing speed, which are often observed following frontal lobe damage, and impaired visuospatial abilities, which are commonly observed following parietal lobe injury. CO-induced brain lesions may occur in the caudate, globus pallidus, cerebellum, hippocampus, and cortical atrophy is also reported.[15,33,48-52] However, in our group of CO-poisoned patients, only one patient had globus pallidus lesions, whereas nine patients had WMH. This finding suggests that lesions in the globus pallidus may occur less frequently than has been reported previously.[34,38] These differences may be due to differences in CO poisoning severity or selection bias.

We found significantly more PVWMH, but not CSWMH, in the CO-poisoned patients than in control subjects, similar to the observations of other investigators (table 2).[13,34,36-38,48,52-57] Thus, our hypothesis was both partially supported in that we found more PVWMH and partially rejected as we did not find more CSWMH in the CO-poisoned patients. WMH are present in 10 to 100% of CO-poisoned patients. In the other CO studies (combined n = 497), the mean number of subjects with WMH was 33% compared with the 12% found in our study. Part of the variability in the frequency of WMH may be explained by 1) the method(s) of patient selection, 2) demographic variables, 3) poisoning variables (mode, COHb levels, CO exposure duration), 4) scanning variables, and 5) time from CO exposure to scanning. For example, one group of CO-poisoned patients (n = 18, mean age 60 years) that was exposed to CO during a coal mine explosion was imaged 25 years after CO exposure.[52]

The strengths of our study include 1) enrollment of prospective consecutively enrolled CO-poisoned patients, 2) lack of subject selection bias, 3) a larger sample size than prior studies, 4) age- and sex-matched normal control subjects, and 5) use of a standardized semiquantitative rating scale[45] that is fast and relatively easy to learn and has a high degree of inter-rater reliability. Unlike previous studies, we did not select patients based on severity, presence of neurologic sequelae, or presence of WMH. Our study prospectively assessed consecutively enrolled CO-poisoned patients who met inclusion criteria. To our knowledge, only one other study assessed consecutively CO-poisoned patients, of which 37% of the patients had WMH.[53] Possible reasons for the difference between these two studies include 1) age difference, 2) CO poisoning severity, and 3) MR scanning methodology such as echo time, repetition time, time to scan, and pulse sequences.

In addition to WMH, CO poisoning can damage other white matter structures.[31-33] Previously, we used quantitative MR to measure white matter atrophy in the fornix and corpus callosum in these same CO patients. We found subtle fornix[32] and corpus callosum[31] atrophy at 2 weeks and 6 months following CO poisoning, with no appreciable atrophic changes on the day of CO exposure. These findings support the idea that CO poisoning may result in damage to cerebral white matter, both acutely as seen with WMH and at some delay after CO poisoning as shown by white matter atrophy in the corpus callosum and fornix.

Table 2 Imaging studies after carbon monoxide poisoning in which white matter changes were observed

Image type	No. of subjects	% with white matter abnormalities	Reference
MRI	15	100	13
MRI	13*	92	52
CT	23	57	54
CT	13	54	55
MRI	30	37	53
CT	60	35	38
CT	129	33	37
CT	18	33	56
MRI	19	32	34
CT/MRI	18	28	57
CT	19	26	48
CT	27	22	17
MRI	73	12	Current study
CT	40	10	36
Total	497	33	

The term "white matter abnormalities" refers to either white matter hyperintensities on MRI or leukoariosis on CT. Studies are listed in descending order by percentage of patients with white matter abnormalities. Only studies with ≥12 subjects are included.

*Mean age = 60 years.

Previous studies have reported increased[58] or decreased[59] WMH over time following CO poisoning. However, we found that the WMH remained consistent from day 1 to 6 months, an observation that is compatible with a previous report that white matter changes appear to be permanent.[37] The WMH observed on the day 1 scan raise the question as to whether these WMH developed before the CO exposure or occurred after CO poisoning but prior to the day 1 scan. White matter signal changes are observed on MR scans between 8 and 24 hours after onset of cerebral ischemia.[60] Alternatively, others have reported late appearance of WMH related to CO-induced delayed encephalopathy.[13] Our CO-poisoned patients' day 1 scans occurred within 24 to 36 hours after CO exposure, and WMH were observed on these scans. It is likely that the white matter changes were due to CO, as the WMH occurred more frequently in the CO patients than in the normal control subjects. The odds of our findings being due to a random event are 1/25 ($p = 0.04$). However, because we do not have pre-CO exposure scans, it is possible that some CO-poisoned patients had WMH prior to their CO exposure.

There were significantly more PVWMH in our CO patients than in the control subjects; however, we found no association between PVWMH and cognitive sequelae. The lack of relationship between PVWMH and cognitive sequelae may be due to the size or location of the PVWMH, inadequate sample size, or tests that were insensitive to cognitive impairments that may have been present. One study reported that the location of PVWMH, particularly in long white matter tracts that connect the cortex with subcortical nuclei or other cortical regions, was related to cognitive sequelae.[3]

Both size and location of PVWMH may account for the lack of correlation with cognitive impairments in our CO-poisoned patients. Another explanation for the lack of association between PVWMH and cognitive performance may be a threshold effect, such that only extensive WMH are associated with cognitive deficits.[5,61] For example, several studies[61] have suggested that WMH fail to affect cognition until the WMH exceed a threshold of 10 cm^2; alternatively, another study[62] indicates that the WMH had to exceed 0.5% of intracranial volume. These thresholds are roughly equivalent to a rating of 2 on the semiquantitative rating scale.[45] Only one of our patients (age 86) had a WMH rating of 1.0, with the rest having a rating of 0.5, representing small WMH. Therefore, it may be that the size of the PVWMH in our sample was too small to detect cognitive impairment by the neuropsychological tests we administered.

Although the CSWMH were related to slower processing speed and cognitive sequelae, CO-poisoned patients did not differ from control subjects in regard to the presence of CSWMH. In addition, a number of CO patients had cognitive sequelae but no detectable white matter changes. Because there were no differences in CSWMH between the CO-poisoned patients and control subjects, we cannot be certain that the CSWMH were caused by CO poisoning. Further research will be necessary to determine if the cognitive sequelae are related to CSWMH that occur after CO exposure or whether the observed association may be due to other risk or vulnerability factors.

We found no relationship between WMH and markers of poisoning severity such as initial COHb levels, loss of consciousness, duration of unconsciousness, and duration of exposure. Other studies have found no relationship between markers of poisoning severity, symptoms of poisoning, or cognitive sequelae.[15] Although duration of unconsciousness correlated with several of the day 1 cognitive measures, loss of consciousness did not correlate with any cognitive measures at 6 months. The duration of unconsciousness may be a marker of acute cognitive impairments but may not predict long-term cognitive sequelae.

The limitations of our study include the following: 1) A small number of patients had WMH. 2) We enrolled CO-poisoned patients with a range of poisoning severity, not just those with severe CO poisoning. 3) Physiologically significant WMH may have been underestimated owing to imaging parameters that may be more sensitive in assessing white matter integrity. 4) Most of the WMH observed in our study were small, and there may be a threshold below which the degree of WMH makes a negligible difference to cognition.

CO poisoning can result in brain injury manifested by WMH and cognitive sequelae. Further research is needed to determine the extent of the severity of the white matter changes that occur following CO poisoning.

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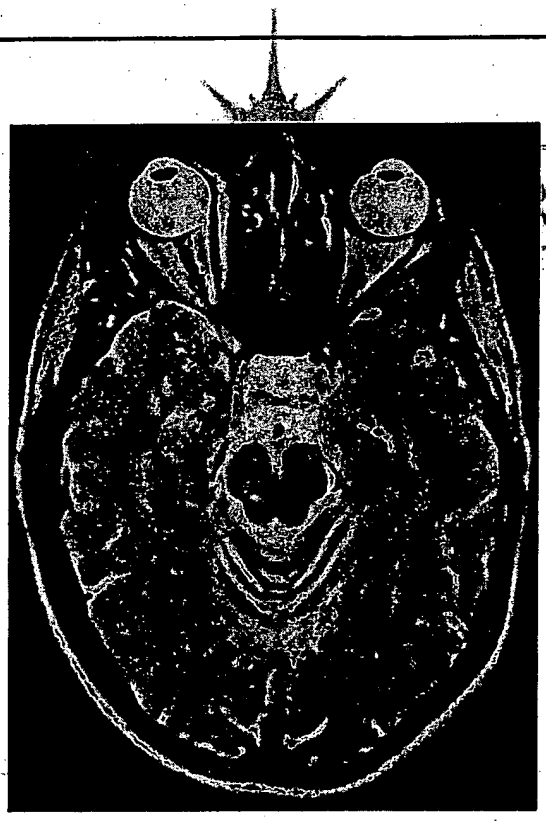

Figure. Axial T2 MRI at the level of the caudal midbrain demonstrates a hyperintense lesion at the posterolateral border of the red nucleus, close to the right third nerve fascicle. White-matter lesions around the right temporal horn, and in the occipital lobes, are also seen.

Neuro *Images*

Painful third nerve palsy in MS

P.I. Bentley, MRCP, D. Kimber, FRACP, A.H.V. Schapira, MD, DSc, FmedSci, London, United Kingdom

A 36-year-old woman presented with an acute, painful, pupil-involving third nerve palsy. She had been diagnosed with MS 5 months previously, when she had presented with a resolving myelopathy. MRI scans at that time had shown multiple white matter lesions in the brain and spinal cord consistent with demyelinating plaques. On this occasion, repeat MRI brain scan showed a new midbrain lesion adjacent to the right third nerve fascicle (figure). MRA of the circle of Willis was normal. The patient was treated with IV methylprednisolone and made a full recovery. Isolated third nerve palsy is rare in MS,[1] but may mimic a posterior communicating artery aneurysm.[2]

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